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OPTIMIZING HER2 ANTIGEN PRESENTATION IN THE MHC I

Trisha Rene Sippel
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OPTIMIZING HER2 ANTIGEN PRESENTATION IN THE MHC I

By

Trisha Rene Sippel

THESIS

Submitted to
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2009

SIGNATURE APPROVAL FORM

This thesis by Trisha Rene Sippel is recommended for approval by the student's thesis committee in the Department of Biology and by the Dean of Graduate Studies.

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ABSTRACT

OPTIMIZING HER2 ANTIGEN PRESENTATION IN THE MHC I

By

Trisha R. Sippel

HER2 is overexpressed in some breast cancers, leading to an aggressive form of the disease. Recent research has targeted HER2 overexpressing tumor cells through the activation of cytotoxic T cells. To do this, HER2 antigens must be cross-presented from the extracellular environment into the MHC I. To measure cross-presentation, a variation of HER2 (EVI) which contains the sequence SIINFEKL was presented to splenocytes. If the splenocytes present SIINFEKL in the MHC I, they will activate the cytotoxic T cell line (B3Z), which is specific to SIINFEKL presented in the MHC I. In attempt to increase cross-presentation, EVI was glycosylated by conjugation to oxidized mannan. The glycosylated EVI demonstrated an increased cytotoxic T cells response when compared to the non-glycosylated EVI, as measured by activation of B3Z cells through production of β -galactosidase. The β -galactosidase production was also greater than that seen in response to the SIINFEKL peptide itself. In another attempt to increase cross-presentation of whole protein, antigens were mixed with commercial transfection agents. The transfection agent PULSin was able to increase β -galactosidase production in response to ovalbumin. This result indicates that transfection agents may be useful for increasing cross-presentation. This study provides insights into the optimal antigenic conditions to consider when creating a tumor vaccine against HER2.

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DEDICATION

I would like to dedicate this work to my Aunt Cindy, Aunt Jane, and Grandma Sippel, who were lucky enough to win the fight against breast cancer; and in memory of my high school German teacher, Frau Matusenic.

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This thesis follows the format prescribed by the journal *Oncogene* and the Northern Michigan University Department of Biology.

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LIST OF ABBREVIATIONS AND ACRONYMS

° C – Degrees Celsius	14
# – Number	23
x g – Standard Gravity	15
[³ H]-thymidine –Tritium labeled thymidine.....	12
μg – Microgram	21
μL – Microliter	14
μm – Micrometer	16
μM – Micromolar	25
APC – Antigen Presenting Cell	8
ATP – Adenosine Triphosphate.....	95
B Cell – Bone Marrow-Derived Cell.....	34
C57BL/6 – Male Mouse 57 Black 6 (Denotes Original Breeding Stock)	22
CD4 – Cluster of Differentiation Molecule 4.....	9
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cm ² – centimeters squared	22
CO ₂ – Carbon Dioxide.....	22
CTLA-4 – Cytotoxic T Lymphocyte antigen-4	8

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<i>E. coli</i> – <i>Escherichia coli</i>	12
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EDTA – Ethylenediaminetetraacetic Acid	20
EGFR – Epidermal Growth Factor Receptor.....	1
ELISA – Enzyme Linked Immunosorbant Assay.....	27
ER – Endoplasmic Reticulum.....	34
ErbB – Epidermal Growth Factor Receptor	1
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EVIrGM – EVI conjugated to GM-CSF.....	36
FBS – Fetal Bovine Serum	22
FITC – Fluorescein Isothiocyanate Conjugate	30
FPLC – Fast Protein Liquid Chromatography	16

g – Grams	14
GM-CSF – Granulocyte Macrophage Colony Stimulating Factor	36
GuHCl – Guanidine Hydrochloride	16
HBSS – Hank’s buffered salt solution	27
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	22
HER – Human Epidermal Growth Factor Receptor	1
HRP – Horseradish Peroxidase	65
hrs – Hours	14
Ig – Immunoglobulin	4
IL-2 – Interleukin-2	45
IMDM – Iscove’s Modified Dulbecco’s Medium	24
IPTG – Isopropyl β -D-1-Thiogalactopyranoside	15
L – Liter	14
LB – Luria-Bertani	14
LDS-PAGE – Lithium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis	18
M – Molar	15
MAP – Mitogen Activated Protein	1
MgCl ₂ – Magnesium Chloride	15
MHC – Major Histocompatibility Complex	7

min – Minutes	15
mL – Milliliter	14
mm – millimeter.....	29
mM – Millimolar	15
MOPS – 3-Morpholinopropane-1-Sulfonic Acid	19
MPER – Mammalian protein extraction reagent.....	25
MUC1 – Mucin 1	11
MWCO – Molecular Weight Cut Off	21
NaCl – Sodium Chloride	15
neu – Name Derived from a Neuroglioblastoma Murine Cell Line	13
NF-AT – Nuclear Factor of Activated T cells.....	52
NEAA – Non-essential amino acids	22
NiSO ₄ – Nickel Sulfate	17
nm – Nanometer	17
N-extended – Amino-terminus extended.....	3
N-linked – Nitrogen-linked.....	12
O-linked –Oxygen-linked.....	12
OVA – Ovalbumin.....	36
p – Probability Value	33

PBS – Phosphate Buffered Saline.....	20
PEI – Polyethyleneimine (PEI)	93
pH – Potential of Hydrogen	15
PI3K – Phosphoinositide-3 Kinase.....	2
PKC – Protein Kinase C	45
PMA – Phorbol 12-Myristate 13-Acetate	27
RNA – Ribonucleic Acid	3
rpm – Revolutions per Minute.....	22
RPMI – Roswell Park Memorial Institute Media	22
SDS – Sodium Dodecyl Sulfate	19
SIINFEKL – Serine-Isoleucine-Isoleucine-Asparagine-Phenylalanine-Glutamate-Lysine- Leucine.....	35
siRNA– small interfering RNA	3
T Cell – Thymus-Derived Cell	7
TCR – T cell Receptor	35
TAP – Transporter Associated with Antigen Processing	35
β-gal – β-Galactosidase.....	35

LITERATURE REVIEW

Human Epidermal Growth Factor Receptor 2 (HER2) is a protein receptor found on most cells in the body (Bernhard *et al.*, 2002). It facilitates the regulation of cell growth and proliferation through interactions with its HER family members. When overexpressed on the cell surface, the cell can become hyper-sensitive to growth factors, or the receptor may become activated without binding a growth factor, causing the cell to grow and divide out of control (Moasser, 2007a). This overexpression of HER2 has been shown to be the cause of 25-30% of all breast cancers (Slamon *et al.*, 2001). Because HER2 is a self-protein, these cancer cells then go unrecognized by the immune system, making HER2 overexpressing breast cancer an aggressive form of the disease. The presence of HER2 overexpression has been correlated with decreased survival and a faster progression of the disease when compared to those with breast cancer not overexpressing HER2 (Slamon *et al.*, 2001). For this reason, HER2 has become a molecular target for breast cancer therapy.

In its natural form, HER2 belongs to a family of four epidermal growth factor receptors: epidermal growth factor receptor (EGFR, HER1, erbB1), HER2 (erbB2, HER2/neu), HER3 (erbB3), and HER4 (erbB4). The HER family proteins are transmembrane tyrosine kinase receptors which upon binding of a ligand to the extracellular domain will undergo either heterodimerization with one of the other HER family proteins or homodimerization with its equivalent HER family protein. This dimerization induces transphosphorylation of the intracellular domain, activating the

receptor (Moasser, 2007a). Signaling molecules are subsequently able to bind the phosphorylated tyrosine residues, activating downstream second messenger pathways such as MAP kinase and PI3K/Akt. Both help signal the cell for proliferation and survival (Hudis, 2007). Akt especially is involved with many pathways, acting as an initiator for several different signals that regulate cellular functions important for the formation of cancer cells. Some of these functions include epithelial-mesenchymal transition, invasiveness, genome stability, cell cycle control, and angiogenesis (Moasser, 2007a).

Of the HER family, HER2 has the strongest catalytic kinase activity, giving it the strongest signaling capability when dimerized with other HER family proteins. Unlike the other HER family proteins, HER2 does not contain a ligand binding domain. As a result, its extracellular domain does not switch between an active and inactive conformation, but instead exists in a constitutively activated conformation. When the HER2 gene becomes overexpressed, the amplification of the receptor on the cell surface can cause HER2 to form homodimers, which do not usually occur under normal expression (Garret *et al.*, 2003). With its constitutively active conformation and strong signaling ability, overexpression of HER2 leads to increased stimulation of its signaling pathways, resulting in uncontrolled cell proliferation and survival.

The overexpression of the HER2 protein can occur either through amplification of the gene itself, or through deregulation of transcription within the cell (Slamon *et al.*, 1989). Breast cancer tumors have been found to contain anywhere from 25 to 50 copies of the HER2 gene per cell, causing a 40-100 fold increase in HER2 protein

expression and resulting in up to 2 million receptors on the cancer cell surface. When HER2 is overexpressed on a cancer cell, the overabundance of growth signaling, along with subsequent evasion of signaling control, is usually the initial cause of the cancer. HER2 amplification is also maintained throughout disease progression and metastasis. Therefore, HER2 amplification is a subtype of breast cancer, rather than an indication of a later stage (Moasser, 2007a).

Initially it was proposed that a mutation in the HER2 gene could form a mutated HER2 protein that would then be constitutively activated, causing continuous downstream signaling of proliferation without dimerization. Using rodent models, it has been demonstrated that a single base pair mutation causes a truncated version of the HER2 protein that is missing the extracellular domain and is constitutively active. This mutation has not been found in humans. It has been predicted that the human form of the protein would require a two base pair substitution, which is much less likely to occur than a single base pair mutation (Moasser, 2007b).

Just as HER2 is responsible for the initiation of tumorigenesis, it is also required for the continuation of tumor growth. This dependency of the tumor on HER2 expression has been termed 'oncogene addiction'. Without the overexpression of HER2, the tumor cells would no longer grow out of control. Cell culture experiments have demonstrated that the knockout of HER2 by siRNA in cancer cell lines overexpressing HER2 results in cell death. Tumors not overexpressing HER2 however are not responsive to HER2 knockdown (Moasser, 2007a).

A better understanding of HER2's transforming ability and aptitude to maintain tumor proliferation has come with the study of cancer stem cells. These cancer stem cells express properties similar to normal stem cells in that they are able to self renew and also differentiate into specific cell types. They are therefore thought to be the tumor-initiating cells, driving the uncontrolled growth of the tumor by deregulating their self-renewal pathways. Korkaya *et al.* (2008) demonstrated that HER2 amplification regulates the mammary stem/progenitor cell population to drive tumorigenesis. They showed that HER2 overexpression on both normal mammary epithelial cells and malignant mammary cells causes an increase in the stem/progenitor cell population as identified by the stem cell marker aldehyde dehydrogenase (Korkaya *et al.*, 2008).

As a result of HER2 overexpression driving tumorigenesis and invasiveness, and the tumor's dependence on it for survival, HER2 is an excellent target for breast cancer therapy. One successful approach at targeting HER2 was the use of the monoclonal antibody trastuzumab. Trastuzumab, a humanized mouse monoclonal antibody, is derived from the combination of a murine antigen binding domain specific to HER2 with the framework region of human IgG (Carter *et al.*, 1992). A phase III clinical trial of the monoclonal antibody in combination with the standard chemotherapy treatment for breast cancer correlated to a longer time to disease progression, higher rate of objective response, longer duration of response, and lower death rate at one year.

Women who had relapsed after chemotherapy also had a greater response when a different chemotherapy was combined with trastuzumab compared to the new chemotherapy alone. In addition, women who had significantly greater amplification of

HER2 benefited more from the addition of trastuzumab to their chemotherapy treatment when compared to women who had HER2 overexpression to a lesser extent, indicating the specificity of trastuzumab to inhibiting HER2 activity (Slamon *et al*, 2001). After this phase III trial, Trastuzumab (more commonly known as Herceptin) was approved by the FDA for treatment of HER2 breast cancer and is now considered a standard therapy.

The side effect of trastuzumab treatment of greatest concern, especially in combination with anthracycline chemotherapy treatment, is cardiac dysfunction. Several other side effects were observed during the phase III trial; however, none were severe enough to cause the discontinuation of treatment. Cardiac dysfunction, on the other hand, was the cause of discontinuation for eight percent of the participants (Slamon *et al.*, 2001). A larger study indicated the risk of cardiotoxicity to be 4.7% in patients receiving trastuzumab (Eisenhauer, 2001). Evidence suggests that cardiac dysfunction, as a result of trastuzumab treatment, occurs from the presence of HER2 receptors or a related cross-reactive antigen on cardiac muscle. When patients with HER2 positive breast cancer were treated with radiolabeled trastuzumab, myocardial uptake was observed, followed by subsequent cardiotoxicity. The antibody therefore was not only targeting the tumor cells, but also the myocardium (Behr, 2001).

In order to make the treatment more effective and avoid the side effect of cardiac dysfunction, it is important to understand the mechanism behind which trastuzumab acts on HER2. Several different mechanisms of action have been predicted for trastuzumab. When the antibody binds the extracellular domain of HER2, it may

block dimerization of HER2 with any of the other HER receptors. It could also cause an increase in the endocytotic destruction of the receptor, or prevent shedding of the extracellular domain of HER2. Shedding of the extracellular domain has been linked to autophosphorylation of the intracellular domain and therefore activation of the secondary messenger pathways. It has also been suggested that binding of the antibody to the receptor could induce the activation of cell mediated cytotoxicity which would then lead to destruction of the cancer cell by immune effector cells (Hudis, 2007).

Understanding the mechanism by which trastuzumab works may also help develop additional strategies for targeting HER2, especially for HER2 overexpressing tumors that are resistant to trastuzumab. While trastuzumab has proved to have a significant impact on treatment of HER2 overexpressing breast cancer, a relatively large fraction of patients treated with adjuvant chemotherapy still relapse, one-third of patients with advanced forms of the disease fail to respond, and the majority of initial responders have disease progression within one year (Korkaya *et al.*, 2008). In order to increase the benefit of HER2 therapy, other methods of targeting the molecule are being developed.

One area of focus involves the final proposed mechanism of action for trastuzumab involving an immune effector response. Instead of using a monoclonal antibody to target the receptor for immune destruction, attempts have been made to develop a vaccine against HER2 through presentation of the protein to antigen presenting cells. Not only would this method possibly target HER2 cancer cells, but it might also create an immunological memory that could prevent relapse after the initial

therapy. It would have the advantage of creating an immune response to a protein already present in the body, but only target those cells that overexpress the protein, not those that have the protein at basal levels (Bernhard *et al.*, 2002).

When creating a vaccine against a self-protein, such as HER2, there is concern that an immune response will not be mounted against the antigen because the T cells specific to the antigen presented in the vaccine would already be eliminated to avoid self-reactivity. It was observed that patients with HER2 overexpression naturally have a pre-existing immune response to HER2, both in the form of antibodies and T-cell immunity, showing that not all T cells specific to HER2 have been eliminated, and making it a possible candidate for use in a vaccine. The immune response was found only in patients who had overexpression of HER2, and was of very low magnitude (Bernhard *et al.*, 2001). This pre-existing response showed no evidence of autoimmune disease, indicating the immune response did not attack cells with basal expression of HER2, and further supporting the development of vaccines in attempt to boost the immunity of these patients.

Normally, to avoid self-reactivity, when a self-protein is processed there are certain dominant antigens that are presented due to the breakdown of the protein and combination of the peptide fragments with major histocompatibility complexes (MHCs). These dominant antigens prevent activation of the immune system against the self-protein. When the protein is overexpressed however, there is more protein to be processed by antigen presenting cells and other, less dominant, peptide fragments besides the dominant antigens are able to be presented. These other fragments can

then induce an immune response against the protein. This mechanism can be mimicked through a vaccination that presents large amounts of protein to antigen presenting cells to be processed (Bernhard *et al.*, 2002). Since the tumor is overexpressing the protein as well, the vaccine will specifically target the other, less dominant, fragments presented on the tumor cell and not the dominant self antigens on normal cells.

Early results from previous vaccine attempts have shown that immunity could not only be elicited, but remain intact after vaccinations had ended. These kinds of vaccines may not only be used as a therapeutic agent for patients with HER2 overexpressing breast cancer, but may also to prevent remission or even the onset of breast cancer (Bernhard *et al.*, 2002). This suggests that the severe risk of cardiac dysfunction related to treatment with trastuzumab could be eliminated through the use of vaccines and that these vaccines could be used in conjunction with standard therapy for an increased benefit.

Rohrbach *et al.* (2005) produced a vaccine in attempt to target delivery of the HER2 (ErbB2) protein to professional antigen presenting cells (APCs). The vaccine antigen was formed by producing a chimeric protein containing an ErbB2 fragment with the extracellular domain of cytotoxic T-lymphocyte antigen-4 (CTLA-4). CTLA-4 is normally found on T cells and specifically binds to B7 molecules on the APC surface, aiding in internalization of the peptide. This protein was named C-ErbB2 and produced through bacterial expression. Another variation of C-ErbB2 was engineered to also contain the *Pseudomonas* exotoxin A translocation domain, which acts as a cytotoxin to

cause cell death (C-E-ErbB2). These two vaccines were then compared to recombinant ErbB2 (ErbB2₂₂₂) as a control (Rohrbach *et al.*, 2005).

The chimeric proteins were able to bind strongly to the antigen presenting cells and were internalized rather quickly (the C-ErbB2 within one hour), which is an essential step for the generation of peptide epitopes and antigen presentation. When these peptides were injected into mice as a vaccine, the mice were able to reject tumor formation when challenged with ErbB2 tumor cells as compared to mice vaccinated with only PBS. Not only were they able to ward off tumors, but when re-challenged with the tumor cells two months after vaccination the mice that received the C-ErbB2 were still immune to tumor formation (Rohrbach *et al.*, 2005).

This study also showed that tumor rejection was dependent on induction of CD8⁺ effector cells by the protein vaccination. Mice were vaccinated with either C-ErbB2 or C-E-ErbB2 and then injected with CD8- and/or CD4-specific antibodies to deplete these T cell populations. When challenged with the tumor cell inoculation, tumors grew in the mice with depleted CD8⁺ or CD8⁺ and CD4⁺ effector cells to an extent comparable to mice vaccinated with only PBS. The mice with only depleted CD4⁺ cells however were still able to reject tumor formation (Rohrbach *et al.*, 2005). This showed that the CD8⁺ cells were required for tumor rejection. It also suggested that the antigen delivered to the APC was loaded into MHC I, since CD8⁺ T cells recognize antigens presented only in MHC I.

Two important aspects of this study were the discovery that HER2 could be presented to antigen presenting cells and used to form an immune response against

tumor cells overexpressing HER2, and that the antigen was being presented primarily through MHC I, which targeted CD8⁺ T cells. This suggests that if the antigen could be altered in another way to provide better uptake and presentation by the APCs, it could have an even greater effect on targeting HER2 tumors. One possible alteration would be the addition of glycosylated subunits to the protein.

Tan *et al.* (1997) showed that dendritic cells contain a mannose receptor that can help mediate the uptake of antigens to MHC II presentation by detecting the presence of terminal sugars such as mannose, fucose, and N-acetylglucosamine. They showed that mannose receptor-antigen complexes were endocytosed into small coated vesicles and then the peptide alone was transferred to larger vesicles. This suggested the mannose receptor was necessary for internalization, but then released the peptide upon transfer into the larger vesicle. The non-mannosylated antigens were present directly in larger vesicles, suggesting that the non-mannosylated antigens were internalized through a route separate from the mannosylated antigens. Both types of antigen were then shown to be co-localized in vesicles with the MHC II (Tan *et al.*, 1997).

In addition to the improved ability of a mannosylated protein to be internalized compared to non-mannosylated protein, mannosylation also allows more antigen to be presented by the cell. After the receptor complex is endocytosed, the receptor releases its ligand so that it can be processed and transported to the MHC II. The receptor can then be recycled so that more antigen can be bound and endocytosed from the surroundings. The glycosylation of three different T cell peptide epitopes caused a 200-

10,000 fold more efficient presentation of the antigen to induce proliferation of the corresponding peptide specific T cell clone (Tan *et al.*, 1997).

In order for the high concentration of antigen on the APC surface to have an antitumor effect though, the antigen would need to be loaded onto MHC I so that it could target the activation of CD8⁺ cytotoxic T cells that could then kill the tumor cells and prevent the tumor from growing. Since the normal pathway for extracellular antigens to be presented is through the MHC II molecule, cross-presentation of the antigen peptides must occur within the cell in order to be associated with an MHC I molecule. A study by Apostolopoulos *et al.* (1995) showed that the oxidation or reduction of mannan conjugated to an antigen controlled which T cell effectors were activated upon antigen presentation. If the mannan was oxidized, the antigen caused a high activation of cytotoxic T lymphocytes (CD8⁺ T cells) and a low amount of antibody production. Reduced mannan caused the opposite, with a low activation of T lymphocytes and a high production of antibody against the antigen produced (Apostolopoulos *et al.*, 1995). The antigen used in this study was the human MUC1, a protein highly expressed in adenocarcinomas.

These results suggested that oxidation of mannan controlled which pathway the antigen entered after endocytosis. This same group later demonstrated that the oxidized mannan did cause the MUC1 to target the mannose receptor of dendritic cells and enter the MHC I presentation pathway through cross-presentation (Apostolopoulos *et al.*, 2000). This confirmed that glycosylation of a protein to be used as a vaccine, especially through the use of oxidized mannan, can lead to a more efficient

presentation of the antigen peptide through the use of MHC I presentation pathway.

The presentation in MHC I would then target the activation of CD8⁺ cytotoxic T cells for an immune response against cells expressing that antigen.

Burgdorf *et al.* (2007) showed that cross-presentation occurs specifically through the mannose receptor on APCs and endocytosis through the mannose receptor causes the antigen to enter a distinct processing pathway specific to presentation in MHC I. The mechanisms of antigen uptake investigated were pinocytosis, mannose receptor endocytosis, and scavenger receptor endocytosis. They discovered that pinocytosis and scavenger receptor endocytosis placed the antigen in lysosomes that were then specific for presentation in MHC II. The mannose receptor endocytosis however targeted the antigen for early endosomes that were then specific for presentation in MHC I (Burgdorf *et al.*, 2007). These APCs were shown to then activate the appropriate corresponding T cell to the MHC complex in which the antigen had been loaded. This confirmed that mannose receptor uptake is necessary for cross-presentation of an antigen.

Luong *et al.* (2007) used a different approach to show that mannose receptor could be targeted for increased presentation of a glycosylated antigen through antigen protein synthesis in fungi. Fungi preferentially use mannose when creating O- and N-linked glycosylation on their proteins. Taking advantage of this, they also showed that the type of mannosylation affected the immunogenicity of the antigen. Ovalbumin produced in yeast cells (glycosylated) and *Escherichia coli* (non-glycosylated) were presented to bone marrow dendritic cells and tested for presentation through a

[³H]-thymidine incorporation lymphoproliferation assay of CD8⁺ T cells. Not only did the mannosylated OVA cause a higher rate of proliferation of CD8⁺ T cells, but it was observed that OVA containing O-linked mannosylation was more immunogenic when compared to N-linked mannosylation (Luong *et al.*, 2007).

Based on these findings, this project attempted to increase the efficiency of HER2 presentation to APCs by targeting of the mannose receptor through the formation of a glycosylated version of HER2. As suggested by previous results, glycosylation was predicted to increase the cross-presentation of HER2, which would be presented in the MHC I and target activation of cytotoxic CD8⁺ T cells. This project could provide important insight when considering the formation of a novel immunotherapy for HER2 overexpressing breast cancer.

METHODS

Producing EVI in *E. coli*:

*Plating *E. coli* transfected with EVI:*

Luria-Bertani (LB) agar plates were prepared by dissolving 25 g into 1 L distilled water and allowed to solidify. Using a heat sterilized metal loop, a small amount of frozen *E. coli* containing the plasmid EVIpTriEx was streaked onto the plate. The plate was incubated at 37 °C overnight.

*Inoculation of LB broth with *E. coli*:*

A 25% LB broth solution was prepared and heated to a boil. After cooling to room temperature, 25 mL of LB broth was transferred to a 125 mL flask using a sterile pipet. One microliter of carbenicillin (100 mg/mL) per milliliter of broth and 1 µL of chloramphenicol in 50% ethanol (34 mg/mL) per mL of broth was added to the flask. A heat sterilized inoculating loop was used to inoculate the broth with an individual colony from a plate of LB agar with *E. coli* EVIpTriEx streaked on it. The flask was then incubated overnight at 37°C with agitation.

The next morning the overnight growth was used to inoculate 5 larger flasks. One 2 L flask was prepared with 600 mL of LB broth and four 1 L flasks with 350 mL of LB broth for a total of two liters. One milliliter of the growing culture was used to inoculate each flask. The flasks were then incubated at 37 °C with agitation for 4 hrs. After

4 hours each flask appeared cloudy and 1 μ L of 1 M IPTG per mL of broth was added to each flask. The flasks were then incubated with agitation overnight at 37 °C.

EVI inclusion body isolation:

The *E. coli* LB broth inoculations were transferred from the five flasks into five 500 mL centrifuge bottles. The bottles were centrifuged at 3280 x g for 15 min at 4°C in a Sorvall RC 5B Plus centrifuge. The supernatant was removed and the pellets were resuspended into 40 mL of Buffer A (50mM Tris, 25% sucrose, pH 8.0) with protease inhibitors cocktail set III (Calbiochem) (1:1000 dilution) added just prior to use. The cells were then lysed by adding 100 mL of Buffer B (20 mM Tris, 100 mM NaCl, 1% deoxycholate, 1% Triton-X, 0.3 mg/mL lysozyme, pH 8.0) per bottle, with lysozyme (Sigma) and protease inhibitors at a 1:1000 dilution added just prior to use. The suspensions were mixed for 15 min at room temperature. After mixing, 1 μ L of 1 mM $MgCl_2$ per mL solution and 0.02 μ L of benzonase (Novagen) per mL of solution were added to each bottle. The bottles were mixed for at least 10 more minutes or until the viscosity visually decreased.

The solutions from the five tubes were then combined into two 500 mL centrifuge bottles, each with equal volumes. The 500 mL bottles were then centrifuged at 3280 x g for 15 min at 4°C. The supernatant was discarded by pipetting and the pellet was resuspended into 25 mL of Buffer C (20 mM Tris, 100 mM NaCl, 0.5% Triton-X, pH 8.0). More Buffer C was added until each bottle had approximately 150 mL of suspension. This suspension was centrifuged at 3280 x g for 10 min at 4 °C. The

resulting pellet was washed two more times with Buffer C. The supernatant was again discarded and the pellet was this time resuspended into 25 mL of Base Buffer (20 mM Tris, 100 mM NaCl, pH 8.0). More Base Buffer was added until each tube had 250 mL total volume. The bottles were centrifuged at 3280 x g for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended into 10 mL Base Buffer. The resuspended pellet was transferred to a 35 mL Oak Ridge centrifuge tube. The original bottle and transferring pipet were washed with an additional 20 mL of Base Buffer. This was then added to the 35 mL Oak Ridge tube. The two Oak Ridge tubes were centrifuged at 11,000 x g for 10 min at 4 °C. The supernatant was discarded and the pellets were stored at -20 °C in the Oak Ridge tubes until further processed.

FPLC of EVI inclusion bodies:

The pellets in the Oak Ridge tubes were resuspended into 20 mL of solubilization buffer (20 mM Tris, 0.5 M NaCl, 6 M GuHCl, 10 mM imidazole). The tubes were mixed for one hour at room temperature to allow for solubilization of the inclusion bodies. The tubes were centrifuged at 17,500 x g for 10 min at 4°C. The supernatant, which contained the solubilized *E. coli* proteins, was removed and filtered through a 0.22 µm bottle top filter (Corning). This solution was then used for fast protein liquid chromatography (FPLC).

The FPLC apparatus was prepared for purification of the EVI by first loading 100 mM sterile filtered NiSO₄ into a 5 mL HiTrap chelating column (GE Healthcare). The

column was then washed with 15 mL of solubilization buffer. Any protein in the column was eluted by washing the column with 15 mL elution buffer (20 mM Tris, 0.5 M NaCl, 6 M GuHCl, 300 mM imidazole). The column was again washed with 25 mL solubilization buffer while the solubilized *E. coli* proteins were loaded into a 10 mL loop. After the 25 mL of solubilization buffer had run through the column, the protein solution was pushed from the loop into the column and 10 mL samples were collected on a fraction collector. After loading the column with protein, the column was washed with 1.5 mL of solubilization buffer while the loop was loaded with 10 additional mL of the solubilized *E. coli* proteins. This was then pushed from the loop into the column. The column was washed with another 15 mL of solubilization buffer. The buffer was changed to a 20 mM imidazole solution (a mixture of 3.5% elution buffer and 96.5% solubilization buffer) and the column was washed with 15 mL. A fraction collector was used to collect 1.0 mL samples of the 20 mM imidazole column elution during this time. The column was washed with 15 mL elution buffer (300 mM imidazole) to elude the EVI off the column while continuing to collect 1 mL fractions. An additional 15 mL solubilization buffer was pushed through the column.

During the FPLC, a chromatograph was obtained, showing the absorbance at 280 nm of the solution eluted from the column and collected by the fraction collector. The peaks on the chromatograph indicate fractions containing protein. The fractions were then combined into groups containing protein from the different steps in the procedure: flow through (proteins that did not stick to the column, but flowed through when the protein solution was injected into the column), 20mM imidazole fraction

(from the mixture of solubilization and elution buffers that would elute loosely bound proteins), 300mM imidazole fraction (from washing with elution buffer, which should elute the tightly bound EVI protein), and after flow (from the last wash with solubilization buffer). Samples from all groups were subjected to SDS-PAGE gel electrophoresis to assure the EVI was primarily in the 300 mM imidazole fraction.

Gel Electrophoresis of FPLC fractions:

The FPLC fractions were prepared for gel electrophoresis using a protocol to remove the 6 M GuHCl from the proteins. One hundred microliters of the sample was mixed with 900 μ L of 100% ethanol at -20 °C. This was placed at -20 °C for 10 min. It was then centrifuged at 7,200 x g in a table top centrifuge for 8 min. The supernatant was carefully removed using a fine tip pipet. The pellet was resuspended into 1 mL of 90% ethanol at -20 °C and vortexed briefly. This suspension was incubated at -20 °C for 5 min and centrifuged as described for 8 min. The supernatant was again carefully removed. The pellet was allowed to air dry for 5 min and resuspended into 65 μ L distilled water, 10 μ L 500mM DTT, and 25 μ L LDS-PAGE buffer (Invitrogen) for a total volume of 100 μ L. Each sample was heated at 70-80 °C for 10-15 min.

The samples were loaded onto an 8% Tris-Glycine gel (Invitrogen) in a gel box filled with MOPS-SDS buffer (Invitrogen). After the wells were loaded, 500 μ L of NuPAGE antioxidant was added to the upper chamber of the gel box, moving the tip across the chamber as it was expressed. The gel ran for 30-35 min at 200 volts. The gel was removed from the boxed and stained with Simply Blue Safe Stain (Invitrogen) with

agitation overnight. The gel was de-stained with distilled water for several hours, and archived using cellophane sheets and Dry-ease solution (Invitrogen).

Fractions containing EVI were identified by the presence of a well defined band on the gel at around 56,000 Daltons based on protein standards. The fractions containing EVI were combined and prepared for refolding and dialysis.

Estimating protein concentration:

Before dialysis, the combined fractions were tested to estimate their protein concentration using a spectrophotometer. A sample of the combined fractions was diluted 1:10 by mixing 900 μ L of solubilization buffer in a cuvet with 100 μ L sample. Using solubilization buffer as the blank, the absorbance of the sample was taken at 280 nm. The approximate concentration of the sample was then calculated, based on a protein solution with an absorbance of 0.682 at 280 nm having a concentration of 1 g/L.

Dialysis of FPLC fractions/EVI sample:

The combined fractions to be refolded were diluted with Diluent Buffer (20 mM Tris, 6 M Guanidine HCl, 0.5 M NaCl, 2 mM EDTA) to a concentration of 0.3 mg/mL sample in 12 mL total volume. Prior to refolding, DTT was added to the sample to a final concentration of 2 mM in a total volume of 3 mL. For example, when the approximate concentration of the protein was 1.5 mg/mL a volume of 2.4 mL was required for a concentration of 0.3 mg/mL in 12 mL. Therefore, the 2.4 mL of sample was first mixed with 6 μ L 1M DTT, and 594 μ L diluent buffer. This mixture was incubated at 37°C for

30 min. After the incubation, 9 mL of diluent buffer was added to the mixture, bringing the total volume to 12 mL. The sample was then chilled to 4 °C.

The 12 mL sample was injected into a Slide-A-Lyzer Dialysis Cassette (Pierce) with an 18 g needle according to the manufacturer's protocol. The cassette was placed in a float and allowed to dialyze overnight at 4 °C while spinning in renaturation buffer (100 mM Tris, 0.5 M Arginine, 2 mM EDTA, pH 8.0). The buffer was then switched to fresh renaturation buffer and the sample was allowed to dialyze for nine additional hours. The buffer was switched to a phosphate buffered saline (PBS) solution and left to dialyze overnight. The solution was then switched to a fresh PBS solution and allowed to dialyze for an addition eight hours. The EVI solution was removed from the dialysis cassette using an 18 g needle and placed into a sterile 15 mL centrifuge tube. This was stored at 4 °C until the solution was ready to be quantified and concentrated.

Quantifying protein solution:

The concentration of the EVI protein solution, now refolded and in PBS, was found using the Qubit flurometer (Invitrogen) according to the manufacturer's protocol. Four samples were prepared, three standards and one sample of the EVI solution. First, Quant-iT Reagent was diluted 1:200 into Quant-iT buffer to make the Quant-iT working solution. Each sample was then diluted 1:20 in the working solution. The standard samples prepared were at protein concentrations of 0, 200 and 400 µg/mL. Each sample was incubated at room temperature for 15 min. The Qubit was calibrated using the standard samples prepared. The QF value of the EVI sample was then found using

the Qubit flurometer and used to calculate the concentration of the EVI solution according to the following equation:

$$\text{Concentration of sample} = \text{QF value} \times \frac{200}{X}$$

(where X is the volume in μL of the EVI sample diluted in working solution)

Concentrating protein solution:

The EVI solution was concentrated using an Amicon Ultra-15 Centrifugal Filter Device (10,000 MWCO). The filter device was rinsed with PBS prior to centrifugation of the sample by adding 4 mL of PBS to the filter and centrifuging for 15 min at $\frac{3}{4}$ max speed on the Fisher Scientific Centrifuge with a swinging bucket rotor. Any PBS remaining in the filter device was then discarded and the sample was added to the top of the filter device. The sample was centrifuged at $\frac{3}{4}$ max speed for 15 min intervals until the volume remaining in the top of the filter device approached 1.5 mL. It was then centrifuged at $\frac{3}{4}$ max speed for 5 min intervals until there was about 1.0 mL remaining in the device. This was then transferred to a 5 mL syringe and filtered through a 0.22 μm filter into a 1.5 mL sterile tube. The concentration of the EVI was calculated based on the starting concentration, starting volume, and the final volume of the solution. The EVI was then stored at -20°C until it was used as an antigen in the B3Z assay.

B3Z Assay using RPMI media:

Splenocyte preparation in RPMI:

The spleen was removed from a C57BL/6 mouse using aseptic techniques and placed into a 10 mL of Roswell Park Memorial Institute (RPMI) 1640 medium. In a class II biosafety cabinet, two frosted tip microscope slides sterilized in 70% ethanol and washed with sterile PBS were used to homogenize the spleen into the 10 mL of medium, using the rough side of the frosted tips. The medium was transferred to a 15 mL centrifuge tube. The plate was washed with an additional 5 mL of medium, which was added to the 15 mL centrifuge tube. The tube was centrifuged at 1250 rpm in an International Equipment Company Model HN-S centrifuge for 5 min. The supernatant was aspirated and the pellet was resuspended into 1 mL of red blood cell lysing buffer (Sigma). After a one minute incubation in the red blood cell lysing buffer, 14 mL of RPMI medium were added to the tube. The tube was centrifuged at 1250 rpm for 5 min. The supernatant was aspirated and the pellet was resuspended into 7 mL of complete RPMI medium (10% FBS, 1% L-glutamine, 1% kanamycin, 1% NEAA, 1% sodium pyruvate, 1% HEPES, 0.1% 2- β -mercaptoethanol, 0.22 μ m sterile filtered). The cell suspension was transferred to a 25 cm² cell culture flask and incubated for 4-5 hours at 37 °C and 5% CO₂.

Cell counting:

After the 4-5 hour incubation, splenocytes adhering to the plastic were counted using a hemocytometer as described in Barker (1998). The B3Z cells were also counted

in the same fashion before adding them to the plate with the antigen and splenocytes. The cells were prepared for counting by aspirating off the medium in the culture flask and adding 10 mL of new medium to the flask. The flask was scrapped using a cell scraper. The cell suspension was then transferred to a 15 mL centrifuge tube. The suspension was mixed briefly by inverting the tube. A 40 μ L sample was taken from the cell suspension and mixed with 40 μ L of trypan blue (Gibco). The mixture was pipetted onto the hemocytometer and the cells located in the four outer 5 x 5 corner squares were counted. The concentration of the cells in the suspension could then be calculated according to the following equation:

$$\text{Cell concentration} = \frac{\text{\# cells counted on hemocytometer}}{4} \times 2 \times 10,000$$

The cell suspension in the 15 mL centrifuge tube was centrifuged in an IEC Model HN-S centrifuge at 1250 rpm for 5 min. The medium was aspirated off and the pelleted cells were resuspended into the correct volume of medium so that the B3Z cells would have a final concentration of 1.4×10^6 cells/mL and the splenocytes would have a concentration of 2.6×10^6 cells/mL.

Plate set-up:

A 96 well plate was set up with a serial dilution of antigen, starting with the highest concentration in row A. The antigen was diluted to the highest concentration in PBS, to a total volume of 200 μ L. The wells in rows B-H were then filled with 100 μ L of PBS. The serial dilution was started by taking 100 μ L from row A and mixing it with the

100 μ L of PBS in row B. This was continued through row G. Row H was then left with only 100 μ L of PBS as a negative control. Each well had 50 μ L of splenocyte cell suspension and 50 μ L of B3Z cell suspension at the concentrations listed above added. The plate was incubated for 24 hours at 37 °C and 5% CO₂ before preparing it for a β -galactosidase luminescent reading.

B3Z Assay super-activating splenocytes:

Splenocyte Preparation in AIM-V medium:

Splenocytes prepared in AIM-V medium were initially isolated in the same process as described for the RPMI preparation. The only difference in the procedure was to use AIM-V medium throughout the process and then complete serum free AIM-V medium (1% L-glutamine, 1% Penicillin-Streptomycin, 1% NEAA, 1% Sodium Pyruvate, 1% HEPES, 0.1% β -mercaptoethanol, 0.22 μ m sterile filtered) for the final resuspension before transferring the splenocytes to the culture flask for incubation.

Plate set-up:

The B3Z assay using AIM-V and Iscove's Modified Dulbecco's Medium (IMDM) was set up using the same serial dilution of antigen as described in the B3Z assay using RPMI medium. Each well containing antigen and PBS had 5 μ M ionomycin added to it. Finally, 100 μ L of splenocytes at a concentration of 1.3×10^6 cells/mL in complete serum free AIM-V medium was added to each well. The plate incubated for 48 hours at 37 °C and 5% CO₂.

The plate was prepared for the addition of B3Z cells by centrifuging at 1250 rpm for 5 min in an IEC Model HN-S centrifuge. The cells were washed twice with 100 μ L of IMDM medium. After centrifuging the plate again for 5 min at 1250 rpm, the supernatant was aspirated off the cells were resuspended into 100 μ L complete IMDM medium (10% FBS, 1% penicillin-streptomycin, 1% NEAA, 1% sodium pyruvate, 1% L-glutamine, 1% HEPES).

B3Z cells in complete IMDM medium were then counted as described above, and 100 μ L of the B3Z cell suspension at a concentration of 7.0×10^5 cells/mL was added to each well containing 100 μ L splenocytes in complete IMDM medium. The plate was then incubated for an additional 24 hours at 37 °C and 5% CO₂ before preparing it for a β -galactosidase luminescent reading.

β -galactosidase Luminescent readings:

The plate was prepared for a luminescent reading by centrifuging for 5 min at 1250 rpm. The supernatant was discarded and the cells were washed with 200 μ L of PBS. This process was repeated one more time. Into each well was added 50 μ L of Mammalian Protein Extraction Reagent (MPER) (Pierce). The plate was shaken gently on a microplate shaker for 5 min. The Clontech Luminescent β -galactosidase detection kit II reaction solution was prepared by diluting the reaction substrate 1:50 into the reaction buffer. The β -galactosidase reaction solution was allowed to warm to room temperature.

After shaking for 5 min, β -galactosidase positive control (Clontech) was added to the plate. The β -galactosidase positive control was first diluted 1:2000 into PBS. This was diluted further, 1:5, into PBS. The final dilution was used to start a serial dilution of β -galactosidase positive control by placing 100 μ L of the final dilution into well A12 on the plate. Wells B12-H12 were filled with 50 μ L of PBS. The dilution was started by taking 50 μ L from well A12 and mixing it into well B12. The serial dilution was continued through well G12. Well H12 contained only PBS.

The β -galactosidase reaction solution was added to the plate by dispensing 150 μ L into each well containing 50 μ L of cells in MPER and each well containing the β -galactosidase positive control. The plate was incubated for 1 hour at room temperature. The plate was then read using a GloRunner microplate luminometer (Turner Biosystems) and the GloRunner DXL software or the Modulus Microplate Reader (Turner Biosystems).

Fixing Splenocytes with Glutaraldehyde:

Splenocytes isolated into AIM-V media, as described previously, were incubated for 4 hrs at 37°C and 5% CO₂. A 96 well plate was set up with serial dilutions of antigen as described for the B3Z assay using RPMI. The splenocytes were allowed to incubate with the antigens for 15 min at 37°C and 5% CO₂. After 15 min, half the wells containing splenocytes at each antigen concentration were fixed with glutaraldehyde, as described by Hosken *et al.* (1989). Glutaraldehyde was prepared fresh by diluting to a final concentration of 2% in Hank's buffered salt solution (HBSS). This was added to the

splenocyte cell media in each well to a final concentration of 0.05%. The plate was then incubated for one hour at 37 °C and 5% CO₂. B3Z cells were then added to the plate in IMDM media as described for the super-activating method and the plate incubated for an additional 4 hours at 37 °C and 5% CO₂ before preparing for a β -galactosidase luminescence reading.

Activating B3Z cells with PMA/ionomycin:

To directly assay for β -galactosidase production in the B3Z cells, phorbol 12-myristate 13-acetate (PMA) and ionomycin were used to activate the B3Z cells as described by Karttunen *et al.* (1992). A 96 well plate was set up with a serial dilution of PMA and ionomycin, starting with 10 ng PMA and 5 μ M ionomycin as the highest concentration. B3Z cells (100 μ L) were then added to each well at a concentration of 7.0×10^5 cells/mL in either complete IMDM media, complete RPMI, or cells that had been switched from complete RPMI to IMDM media. The plate was incubated at 37 °C and 5% CO₂ for 4 hrs before preparing for a β -galactosidase luminescence reading.

Enzyme Linked Immunosorbant Assay (ELISA) of splenocytes:

To assay for SIINFEKL presentation in the MHC I without using the B3Z cells, an ELISA was conducted on the splenocytes alone. Splenocytes were isolated into complete serum free AIM-V media as described previously. A 96 well plate was set up with a serial dilution as described for the B3Z assay with RPMI. Splenocytes

(100 μ L) were added to the plate and allowed to incubate for 48 hrs at 37 °C and 5% CO₂. The plate was centrifuged at 1250 rpm and the supernatant discarded. The splenocytes were fixed by adding 200 μ L of 4% formaldehyde to each well and incubating at room temp for 10 min before discarding the formaldehyde. Each well was rinsed three times with 200 μ L PBS. The wells were blocked by washing with goat serum diluted 1:20. The goat serum (100 μ L) was added to each well, and incubated for 5 min at 37 °C and 5% CO₂ before washing two times with 100 μ L PBS. This blocking process was repeated two more times. One hundred microliters of the primary antibody (25-D1.16, anti-mouse MHC I bound to the peptide SIINFEKL, diluted 1:1000) was added to each well. The plate incubated 60 min at 37 °C and 5% CO₂.

The plate was washed three times with 100 μ L PBS, before adding 100 μ L of horseradish peroxidase Avidin D (diluted 1:500) to each well. This incubated 60 min at 37 °C and 5% CO₂. The plate was again washed three times with 100 μ L PBS. The secondary antibody was detected using the Thermo Scientific Super Signal ELISA Pico Chemiluminescent Substrate according to the manufacturer's protocol. The substrate (100 μ L) was added to each well. The plate was mixed for one minute on a plate shaker. The luminescence was read on the Modulus Microplate Reader (Turner Biosystems).

Immunofluorescence of B3Z cell T cell Receptor:

Plating B3Z cells:

A 24 well plate was set up with BD BioCoat Poly-L-Lysine 12 mm round coverslips (BD Biosciences) in wells 1 and 2 of rows A and B. The wells were washed with

70% ethanol and then PBS. Two flasks of B3Z cells (one with the old cells that had been in culture in the lab for over a year and the other with the new B3Z cells received from the University of California at Berkeley, courtesy of Dr. Nilabh Shastri) were scraped and transferred to a 15 mL centrifuge tube. The cells were centrifuged for 5 min at 1250 rpm. The supernatant was discarded and the cell pellets were resuspended into 10 mL of complete RPMI. Depending on the size of the pellet formed, either 150 μ L or 300 μ L of cell suspension was added to the wells containing the round cover slips. The final volume in each well was brought to 1 mL with complete RPMI media. The plate was incubated overnight at 37 °C and 5% CO₂.

Measuring Immunofluorescence:

The media was aspirated from each well and 1 mL of 4% formaldehyde was added to fix the cells to the cover slips, incubating for 10 min at room temperature. The formaldehyde was aspirated and the wells were rinsed twice with 1 mL PBS. The wells were blocked with goat serum by adding 25 μ L of goat serum to each well, incubating for 5 min at 37 °C and 5% CO₂, and washing twice with 1 mL BD Pharmingen staining buffer (BD Biosciences). This blocking procedure was repeated two more times. The primary antibody, a mouse monoclonal antibody specific to TCR α and TCR β (Abcam), was diluted 1:100 in staining buffer and 25 μ L was added to each well. The plate was incubated for one hour at 37°C and 5% CO₂.

Each well was washed three times with 1 mL staining buffer over a period of

5 min. The secondary antibody, biotin conjugated anti-mouse IgG (BD Biosciences), was diluted 1:50 in staining buffer and 25 μ L was added to each well. The plate was incubated for an additional hour at 37 °C and 5% CO₂. The wells were washed three times with 1 mL staining buffer over 5 min.

The plate was taken into the darkroom and all subsequent steps were done in the dark. Streptavidin-FITC (BD Biosciences) was diluted 1:25 in staining buffer and 25 μ L was added to each well. Each cover slip was removed from the well and mounted onto a microscope slide with a drop of Vectasheild mounting medium with DAPI (Vector Laboratories). The slides were visualized using an Olympus BX51 Fluorescent Microscope.

Glycosylating Antigens with Oxidized Mannan:

The antigen proteins were glycosylated with oxidized mannan as described by Apostolopoulos *et al.* (1995). Mannan (Sigma) was dissolved to 14 mg/mL in PBS with 0.01 M sodium periodate and incubated for 60 min at 4 °C. Ethylene glycol (10 μ M) was then added to the solution and incubated an additional 30 min at 4 °C. A HiTrap Q Sepharose HP column (GE Healthcare) was equilibrated with 0.2 M sodium carbonate/bicarbonate buffer, pH 9.0. The first 2 mL of void volume was collected. This was mixed with 900 μ g of antigen and allowed to incubate overnight at room temperature. Electrophoresis performed using the non-glycosylated and glycosylated antigens to see if the band for the glycosylated antigen migrated more slowly down the gel due to the added molecular weight of the conjugated mannose.

Protein Transfection Agent delivery of antigens:

TransPass-P protein transfection agent:

Splenocytes were isolated as described previously into serum free AIM-V media. A 96 well plate was set up with 100 μ L of splenocytes at a concentration of 1.3×10^6 cells/mL. The antigens were mixed with the transfection agent TransPass-P (New England BioLabs) as described by the manufacturer. Each antigen was diluted to 10 μ M in serum free AIM-V to a total volume of 30 μ L with 0.6 μ L TransPass-P. This was incubated for 20 min at room temp before adding 10 μ L of the transfection mix to each well containing splenocytes. The plate was incubated at 37 °C and 5% CO₂ for 4 hrs. B3Z cells were added to the plate in IMDM media as described for the super-activating splenocytes procedure. The plate was incubated for an additional 24 hrs at 37 °C and 5% CO₂ before preparing for a β -galactosidase luminescent reading.

PEI (jetPRIME) DNA transfection agent:

Splenocytes were isolated into complete RPMI media as described previously. A 96 well plate was prepared by adding 100 μ L of splenocytes at a concentration of 1.3×10^6 cells/mL to each well. Each antigen was diluted to a concentration of 1 μ g/mL in PBS. To make the transfection mixes, 2 μ L of diluted antigen, 4 μ L jetPRIME (Polyplus-transfection) and 100 μ L jetPRIME buffer were mixed together and incubated at room temperature for 10 min. Five microliters of the transfection mix was then added to a set of wells containing splenocytes. The plate was incubated overnight at 37 °C and 5% CO₂. B3Z cells in complete RPMI (100 μ L) were then added to the plate at a concentration of

7.0×10^5 cells/mL. The plate was incubated an additional 24 hrs at 37 °C and 5% CO₂ before preparing it for a β -galactosidase luminescent reading.

PULSin protein transfection agent:

Splenocytes were isolated into complete RPMI as described previously. Before adding the cells to the plate, they were washed with complete serum free RPMI to remove serum. The splenocytes were then resuspended into complete serum free RPMI media. A 96 well plate was prepared by adding 100 μ L splenocytes at a concentration of 1.3×10^6 cells/mL to each well. To make the transfection mixes, 2 μ L of antigen diluted to 1 μ g/mL, 4 μ L PULSin transfection agent (Polyplus-transfection) and 100 μ L 20mM HEPES buffer were mixed together and incubated at room temperature for 15 min. Ten microliters of transfection mix was added to a set of wells with splenocytes. The plate incubated overnight at 37 °C and 5% CO₂. The plate was centrifuged at 1250 rpm and the supernatant was aspirated off. B3Z cells in complete RPMI media were added to the plate at a concentration of 3.5×10^5 in 200 μ L. The plate incubated for 24 hours at 37 °C and 5% CO₂ before it was prepared for a luminescent reading.

Statistics and Data presentation

Unless otherwise noted, all β -galactosidase luminescent readings are reported as the luminescence value measured for the variable in question with luminescence value of the respective control subtracted from it. The zero line on each graph therefore depicts the luminescence measurement for the respective control. For example, in the

super-activation with ionomycin assays, the luminescence value reported for EVI at each concentration would be the luminescence value measured for EVI on the microplate reader with the luminescence value for the ionomycin control subtracted from it. Statistical difference was then determined by comparing the original measurement for the variable (that did not have the control subtracted from it) and the measurement for the control using a student t-test, with $p \leq 0.05$ considered significant.

CHAPTER 1: B3Z ASSAY USING THE RPMI METHOD

Introduction

Antigen presenting cells of the immune system have two ways of processing foreign materials to initiate an immune response. External antigens, such as cellular debris from infection or tissue damage, are endocytosed and placed into lysosomes. Here the antigen is cleaved into smaller peptides. MHC II molecules produced in the Endoplasmic Reticulum (ER) are transported to the lysosome, where they bind the peptides. This peptide/MHC II complex is then presented on the cell's surface. Internal antigens found in the cytosol, most of which are endogenous to the cell or a result of viral infection, are cleaved by proteosomes in the cytosol. These peptides are then transported into the ER, where they form complexes with an MHC I molecules. The complexes are then transported to the cell surface for presentation (Raghavan *et al.*, 2008).

Peptides presented in an MHC II activate T helper cells, which produce specific cytokines that in turn activate B cells to produce antibodies specific to the peptide that was originally presented in the MHC II. Antigens presented in the MHC I activate cytotoxic T cells, which will then act to destroy any cell displaying the original peptide (Raghavan *et al.*, 2008). This process can be beneficial in the case of tumor antigens. If cytotoxic T cells could be activated to attack tumor antigens found on cancer cells, then these cancer cells could be eliminated by the body's own immune system. In order for

the external tumor antigen to be presented in an MHC I, it must undergo cross-presentation.

Cross-presentation is the process of presenting an external antigen in an MHC I. In order for this to occur, the antigen must be transported into the cytosol. This has been shown to take place through two main types of pathways. In the vacuolar pathway, antigens are brought into the cell by endocytosis in endosomes where they are cleaved into smaller peptide fragments by proteases. These peptides can then be loaded into a recycled MHC I present in the endosome. This complex traffics back to the plasma membrane (Raghavan *et al.*, 2008). The other pathways are known as TAP-dependent. TAP (transporter associated with antigen processing) is found in the ER membrane. In these TAP dependent pathways, antigens are endocytosed but transported into the cytosol where they can be processed by proteosomes and transported into the ER by TAP to be loaded into an MHC I (Raghavan *et al.*, 2008).

To test for cross-presentation, antigens containing the peptide sequence SIINFEKL were placed in the media of antigen presenting cells (splenocytes) in the presence of cytotoxic T cells with T cell receptors (TCRs) specific to the SIINFEKL peptide presented in an MHC I (B3Z cell line) and allowed to process the antigens. If these cells were able to present the peptide SIINFEKL in their MHC I, by cross presenting the antigen, the B3Z cells would be activated. The B3Z cells have been engineered so that the activation of the TCR activates the LacZ gene, resulting in the production of β -galactosidase (β -gal) (Kartunnen & Shastri, 1991).

The antigens used in this assay were: ovalbumin (OVA), which naturally contains the SIINFEKL sequence; EVI, which is composed of the external domain of the HER2 protein, the SIINFEKL sequence, and the internal domain of the HER2 protein; EVIrGM, which is EVI conjugated to granulocyte macrophage colony-stimulating factor (GM-CSF); and EA2-EVI, which is a variation of EVI with an altered external domain. These four antigens were compared for their ability to be presented and activate cytotoxic T cells. EVIrGM was shown to significantly activate the T cells compared to the other antigens. It is predicted that the GM-CSF conjugated to the protein is able to bind the GM-CSF receptor on antigen presenting cells and the antigen would be endocytosed along with the receptor as it undergoes its normal recycling process.

After demonstrating that GM-CSF conjugated to EVI significantly increased cross-presentation, it was hypothesized that GM-CSF alone might increase cross-presentation through increased endocytosis and therefore increase presentation of SIINFEKL. Granulocyte macrophage colony-stimulating factor acts as a growth factor for lymphocytes to promote growth and differentiation. Both OVA and EVI were tested for their presentation in the presence of J558 supernatant. The J558 cell line produces large amounts of GM-CSF.

Methods

RPMI method:

Splenocytes were isolated into complete RPMI media as described previously. After incubating at 37 °C and 5% CO₂ for 4-5 hours, the splenocytes were counted and

resuspended into complete RPMI media. B3Z cells in complete RPMI media were also counted and resuspended. The splenocytes and B3Z cells were added 1:1 to a 96 well plate containing antigens serially diluted as described previously, starting with 10 μ M of antigen. The antigens used were OVA, EVI, EVIrGM, and EA2-EVI. Control wells contained splenocytes, B3Z cells and PBS. Control wells did not contain antigen. The plate was incubated 24 hrs at 37 °C and 5% CO₂, and then the amount of β -gal production was determined using a GloRunner luminometer and the Luminescent β -gal detection Kit II (Clontech).

Serial dilution of GM-CSF:

A 96 well plate was set up with the same concentration of antigen (OVA or SIINFEKL) in each well, but a serial dilution of J558 supernatant, which acted as the source of GM-CSF. Ovalbumin was added to each well at a concentration of 150 μ M in PBS. Twenty-five microliters of J558 supernatant was added to the first wells to start a serial dilution as described previously. A separate plate was prepared with EVI in each well at a concentration of 8 μ M. One hundred microliters of J558 supernatant was added to the first wells to start a serial dilution. Control wells contained either antigen without J558 supernatant, 25 μ L J558 supernatant without antigen, or PBS. Splenocytes and B3Z cells were then added to each well to a 1:1 ratio, bringing the total volume to 200 μ L. The plates were each incubated at 37 °C and 5% CO₂ for 24 hrs and β -gal production was assayed using the Luminescent β -gal detection kit II (Clontech) and the GloRunner luminometer.

Supplementing with GM-CSF:

A 96 well plate was set up with a serial dilution of antigen and equal amount of J558 supernatant. OVA and EVI dilutions were set up starting with 10 μ M of each as the highest concentration. One hundred microliters of J558 supernatant was added to each well. The control wells contained J558 supernatant alone. Splenocytes and B3Z cells were then added 1:1 to each well. The plate was incubated at 37 °C and 5% CO₂ for 24 hrs before the β -gal production was assayed using the Luminescent β -gal detection kit II (Clontech) and the GloRunner luminometer.

Results:

Four different antigens were compared for their ability to activate the cytotoxic T cell line, B3Z. The activation of the B3Z cells was determined by measuring the amount of β -gal production. The β -gal produced by cells presented with each antigen was compared to the β -gal produced by the controls using a student t-test ($p \leq 0.05$ significance). Since the same concentration of cells was not used for each assay, the luminescent reading and antigen concentration are reported per 10⁶ cells.

Three of the antigens showed significant β -gal production for at least two concentrations of antigen. The EVIrGM showed a much larger activation than the other antigens, but was significantly greater than the control at only the highest concentration (Figure 1A). The next largest activation was by the EA2-EVI, which showed significant β -gal production at all concentrations except the lowest (Figure 1A). OVA also showed significant β -gal production at four of the lower concentrations, but did not activate the

cells very much in comparison to the EVIrGM and EA2-EVI (Figure 1B). The HER2 antigen containing the SIINFEKL sequence (EVI) did not have significant β -gal production at any antigen concentration (Figure 1A & 1B).

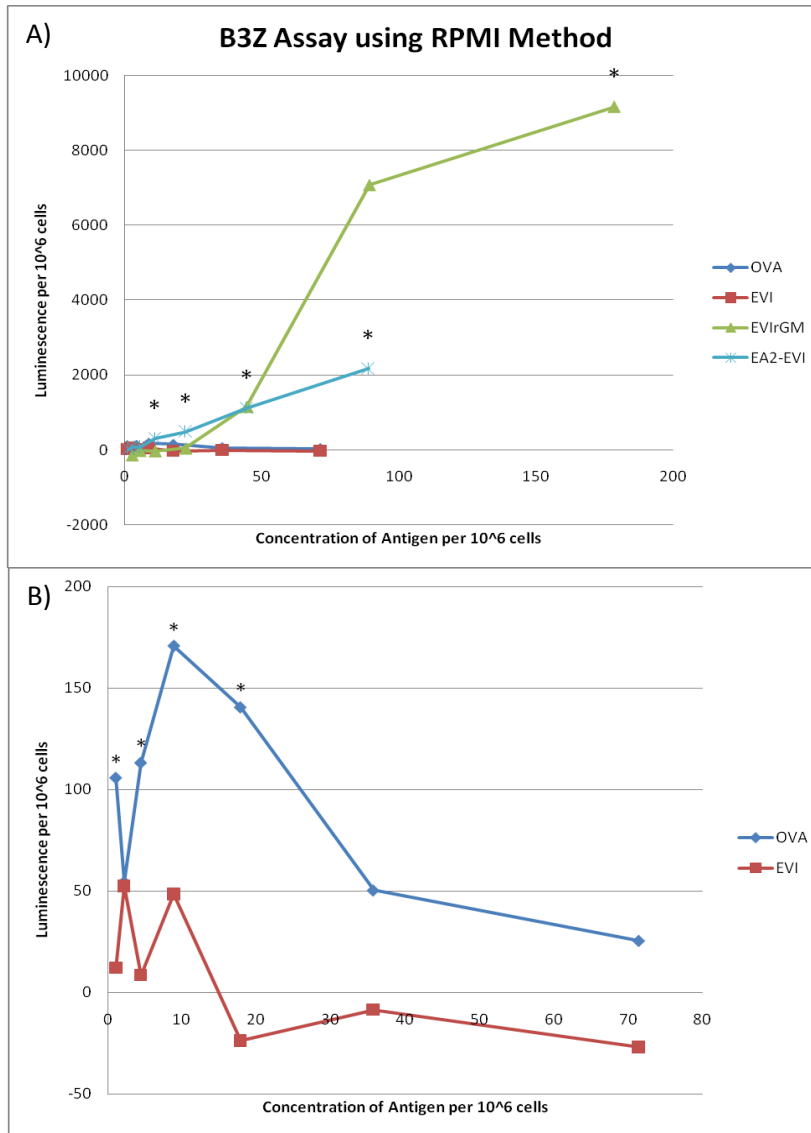
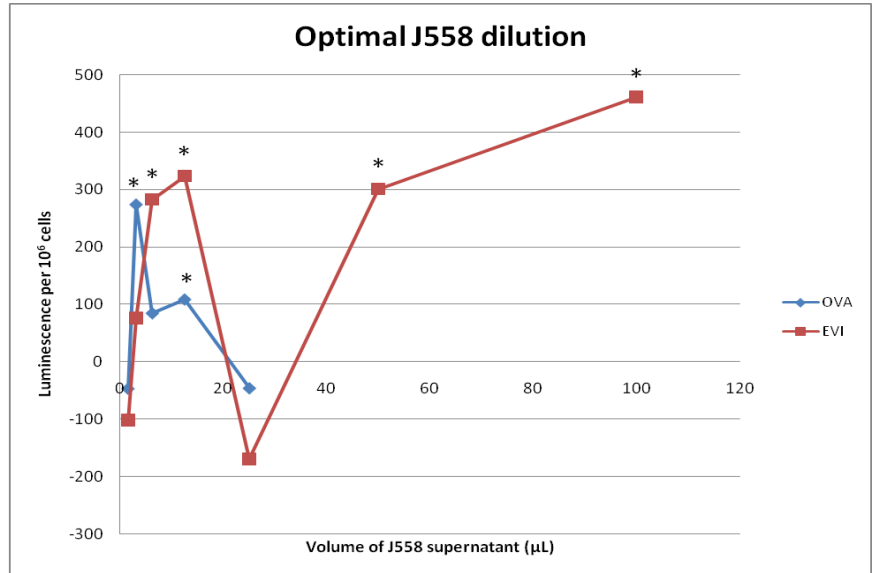


Figure 1: B3Z Assay using RPMI. Splenocytes and B3Z cells were incubated with serial dilution of antigens for 24 hrs. The β -gal production by the B3Z cells as a result of activation by the peptide SIINFEKL presented in a MHC I by the antigen presenting cell was measured by a luminescent assay. The antigens used were OVA, EVI, EVIrGM, and EA2-EVI. All antigens were at a concentration of 10 μ M for the highest concentration. Results are reported per 10⁶ cells, since different concentration of cells were used for each antigen. Luminescent readings significantly greater than PBS control are indicated by the stars (*), determined by a student t-test ($p \leq 0.05$). Panel A shows all antigens, while panel B shows OVA and EVI only.

Since the EVIrGM gave the greatest β -gal production by B3Z cells in response to the splenocytes presented with the highest antigen concentration, an assay was conducted to test if the GM-CSF needed to be conjugated to the EVI, or if it would work as a supplement in the media. The supernatant of J558 cells, collected previously, was added to specific set concentrations of antigen to determine the concentration of supernatant that would aid in activation of the cells by the antigen without activating the cells on its own. Either 8 μ M EVI or 150 μ M OVA was added to each well of a plate with different concentrations of J558 supernatant. The serial dilution of J558 supernatant was then compared to the antigen alone.

Both EVI and OVA showed significant β -gal production at the higher concentrations of J558, with the 100 μ L of J558 supernatant added to the EVI responding the greatest (Figure 2). Different concentrations of cells were used for each assay, so luminescence is reported per 10^6 cells. While the highest concentration of J558 supernatant was able to significantly increase the activation of B3Z cells by EVI, it did not by itself show a significant increase in β -gal production (data not shown). When compared to cells with PBS instead of antigen, 100 μ L of J558 without antigen did not cause significant β -gal production.

Figure 2: Optimal J558 dilution. Splenocytes and B3Z cells were mixed with a set concentration of antigen (8 μ M EVI or 150 μ M OVA) and a serial dilution of J558 supernatant. β -gal production by the B3Z cells, was measured by luminescence per 10^6 cells. Stars (*) indicate significantly greater luminescence than controls ($p \leq 0.05$).



The 100 μ L of J558 supernatant gave the highest response in combination with EVI, and alone did not significantly activate the B3Z cells, so it was used as a supplement with serial dilutions of antigen. OVA and EVI were added to splenocytes and B3Z cells in serial dilutions, starting with 10 μ M of each. One hundred microliters of J558 supernatant was then added to each concentration. Only one concentration of EVI and two of the lower concentrations of OVA showed significant readings when compared to cells with J558 supernatant and no antigen (Figure 3). While these readings were significantly above control, the luminescence per 10^6 cells was much lower than the EVIrGM itself (Figure 1A).

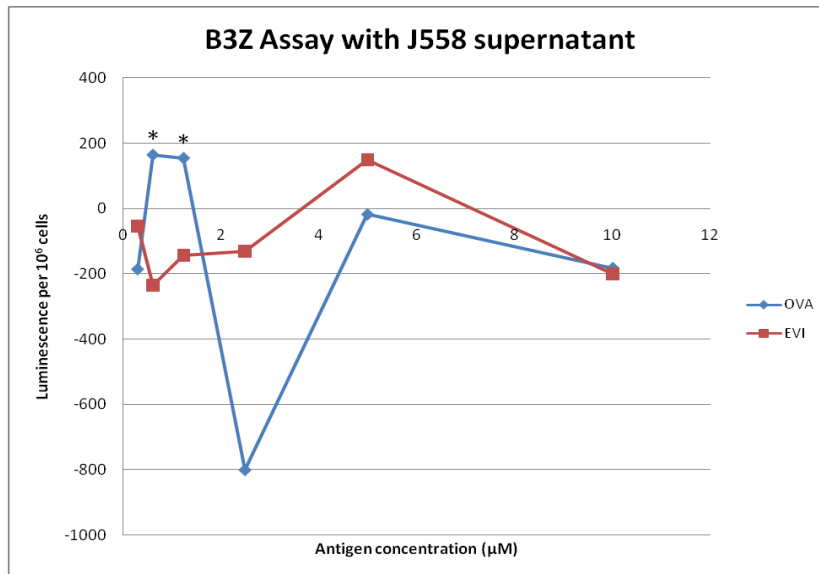


Figure 3: B3Z Assay with J558 supernatant. Splenocytes and B3Z cells were mixed with serial dilutions of OVA and EVI with 100 μL of J558 supernatant per well. Activation was measured by luminescent readings significantly above the control of cells with J558 supernatant alone, as indicated by the stars (*) ($p \leq 0.05$).

Discussion

In order for an external or recombinant antigen to have an effective cytotoxic T cell response, the antigen must undergo cross-presentation into the MHC I pathway. This process occurs in antigen presenting cells through two main pathways. The amount of cross-presentation can be enhanced through alterations of the antigen itself and targeting of receptors on the antigen presenting cell's surface to encourage endocytosis of the antigen. The antigens OVA, EVI, EVIrGM, and EA2-EVI were tested for their efficiency of cross-presentation in the MHC I through activation of the cytotoxic T cell line B3Z.

The antigen which caused the greatest response in B3Z cells as measured by the most β-gal production and therefore the highest luminescent reading was the EVIrGM. The elevated β-gal production by EVI conjugated to GM-CSF may occur because it is able to bind to the GM-CSF receptor and be internalized with the receptor. This would aid in

internalizing the antigen, and therefore increase the probability of undergoing cross-presentation from the external environment into the MHC I. It was demonstrated that the GM-CSF conjugated to the EVI was able to improve cross-presentation when compared to the EVI alone.

While the highest concentrations of EVIrGM showed significant β -gal production, the dose response curve was not quite as expected. Using a serial dilution of antigen, it would be expected that the second highest luminescence would be half the luminescence of the highest concentration, since the concentration of antigen was reduced 50% with each dilution. This pattern was not observed for any of the antigens. This suggests that none of the antigens were presented to their full potential. While some appeared to undergo cross-presentation, none were able to give a response correlated to the amount of antigen presented. The data points themselves also had a large variation, making it hard to draw conclusions about the activation of the T cells by the splenocytes.

In addition to EVIrGM, OVA and EA2-EVI also had a moderate increase in luminescence compared to controls, with some antigen concentrations significantly greater than the control. Ovalbumin acts as a positive control because it is a native protein containing the SIINFEKL sequence. This shows that OVA is able to be processed naturally by the antigen presenting cells and undergo cross-presentation to a certain extent. The EA2-EVI was also able to undergo some cross-presentation, with an even higher efficiency than the OVA itself. This could be due to the extracellular protein

modification, which may have some ability to increase movement into the cell cytoplasm independent of endocytosis.

Following the increased luminescence with EVIrGM, it was tested whether the effect of GM-CSF was only seen when conjugated to EVI. When a serial dilution of J558 supernatant, containing large amounts of GM-CSF, was added to consistent amounts of antigen, it appeared that the more supernatant that was added, the larger β -gal production there was. This may indicate more antigen presentation, or improved cross-presentation, and is likely due to greater activity of the splenocytes. When the highest concentration of J558 supernatant (GM-CSF source) was added to serial dilutions of antigen however, no dose response was seen. This suggests that the GM-CSF may increase cross-presentation when added as a supplement to the media, but is much more effective at increasing cross-presentation of the antigen when conjugated to the protein. The ability of GM-CSF to promote lymphocyte growth was not in itself enough to increase presentation.

The EVI antigen alone was not able to be processed by the splenocytes for cross-presentation as seen in Figure 1. There was not a significant increase in β -gal production at any concentration of EVI. This indicates that while variations of the EVI protein were able to be presented by the antigen presenting cell and activated the cytotoxic T cells, EVI alone was not. The EVI protein would therefore need to be modified in order to make it a productive antigen for a vaccine. One possible method may be to glycosylate the EVI in attempt to increase cross-presentation.

CHAPTER 2: SUPER-ACTIVATION OF SPLENOCYTES

Introduction:

Anti-tumor immunity by antigens specific to the tumor type could be developed to form an effective treatment. In order to optimize the outcome of such a treatment, the tumor antigen would need to be efficiently presented in a high concentration to elicit activation of as many T cells as possible. One way to increase presentation of an antigen would be to have more MHC I molecules available in each cell for binding of peptides produced from the tumor antigen. These peptides in the MHC I would then be able to activate cytotoxic T cells specific to the antigen.

The ionophore ionomycin has been shown to increase the number of MHC I molecules within antigen presenting cells by its ability to induce synthesis of several cytokines. Ionomycin acts to increase calcium influx inside the cell, activates protein kinase C (PKC) pathways, and induces the production of the cytokines IL-2 (Chatila *et al.*, 1989) and interferon- γ (Jabrane-Ferrat *et al.*, 1999). These cytokines are involved with the inflammatory response. In order to increase MHC I production, splenocytes were super-activated with ionomycin. Ionomycin was added to the media and incubated for 48 hours along with the appropriate antigens, allowing for maximal presentation.

Super-activation increases the presentation of antigen, but also increases the length of time that presentation must occur. Hosken *et al.* (1989) showed that it takes as little as 45-60 minutes for OVA peptides to be presented in the MHC I. While the

protein is able to be processed by the antigen presenting cell, and presented on the cell surface in a short amount of time, it is unknown how long the SIINFEKL/MHC I complex will remain on the cell surface. In the super-activation assay the splenocytes are allowed to process and present the antigen for 48 hrs. It is questionable whether the peptide/MHC I remain on the cell surface for that long.

Ludewig *et al.* (2001) demonstrated that the antigen gp33 could be rapidly presented in an MHC I and elicit a cytotoxic T cell response. However, the peptide/MHC I complex presentation did not last very long. One day after presentation of the antigen, a cytotoxic T cell response was no longer able to be activated, suggesting the peptide/MHC I was no longer presented on the surface of the antigen presenting cells (Ludewig *et al.*, 2001). If this is the case for the OVA peptide, SIINFEKL, presented in the MHC I, activation of the B3Z cells may not occur after the splenocytes have incubated with the antigen for 48 hrs, even with the aid of the ionomycin super-activation. A time course of antigen presentation was conducted to determine if antigen incubation time effected the activation of B3Z cells.

Methods:

B3Z assay super-activating splenocytes with ionomycin:

Splenocytes were isolated into serum free AIM-V media as described previously. Splenocytes were incubated with ionomycin and serial dilution of antigen for 48 hours at 37 °C and 5% CO₂ before adding the B3Z cells. The antigens used were OVA, EVI, EVIrGM, and the peptide SIINFEKL. After incubating for an additional 24 hours, β-gal

production was determined using the Luminescent β -gal detection kit II (Clontech) and the GloRunner Luminometer. A serial dilution of β -gal was used as a standard. The luminescence readings, which correspond to β -gal production, taken from the B3Z cells could then be compared to the standard to calculate the amount of β -gal produced. The same β -gal standard serial dilution was used for each plate so that luminescence readings between plates could be compared.

Time course of antigen presentation:

Splenocytes were super-activated in AIM-V media as described previously, but without antigen. After the 48 hour incubation, antigen at the same concentration was added to each of six plates with splenocytes. The splenocytes were then incubated for 1, 2, 4, 8, 24 or 48 hrs with the same concentration of antigen at 37 °C and 5% CO₂ before adding B3Z cells. The antigens used were either 10 μ M OVA or EVI. After adding B3Z cells, the plates were incubated an additional 24 hrs before reading the luminescence on the GloRunner luminometer.

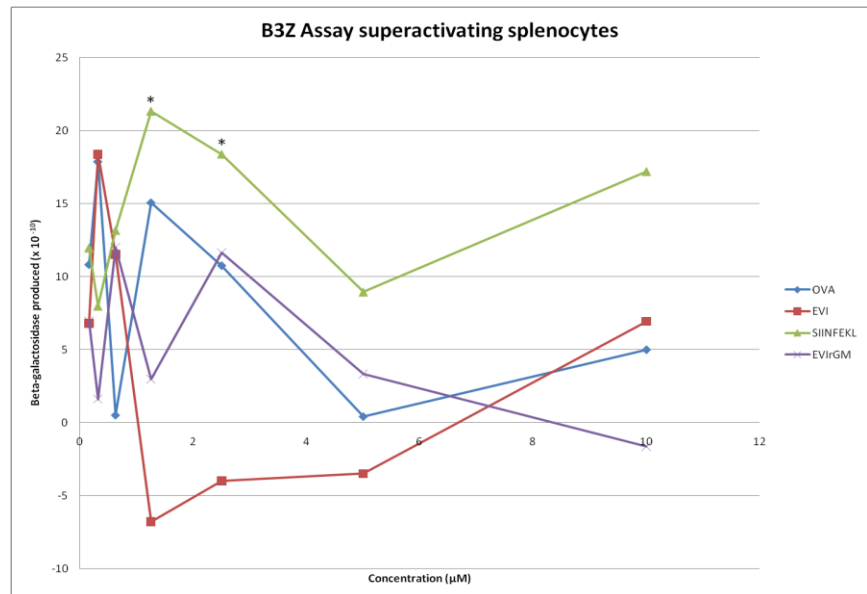
Results:

Splenocytes were super-activated with ionomycin and antigen to determine if antigen presentation could be increased. Ionomycin acts on the splenocytes to increase cytokine production, leading to an increase in the number of MHC I produced by the cell. With an increased amount of MHC I available, the antigen presenting cells may be able to present more peptides and activate more cytotoxic T cells.

Four different antigens were assayed for their ability to activate B3Z cells: OVA, EVI, EVIrGM, and the peptide SIINFEKL. Only the SIINFEKL peptide showed significant activation above the control consisting of cells treated with PBS and ionomycin (Figure 4), but this was only at two of the lower concentrations. EVI and OVA did not show significant β -gal production at any concentration of antigen. Even the EVIrGM, which was able to significantly activate the B3Z cells using the RPMI method (Chapter 1) was not able to produce significant β -gal production using the super-activating method.

Figure 4: B3Z Assay Super-activating splenocytes.

Splenocytes were incubated with ionomycin and serial dilution of the antigens OVA, EVI, SIINFEKL and EVIrGM for 48 hours. B3Z cells were then added for an additional 24 hrs and assay for activation by luminescence. The luminescence was then compared



to a β -gal control to find the amount of β -gal produced. Stars (*) indicate significant β -gal production compared to controls ($p \leq 0.05$).

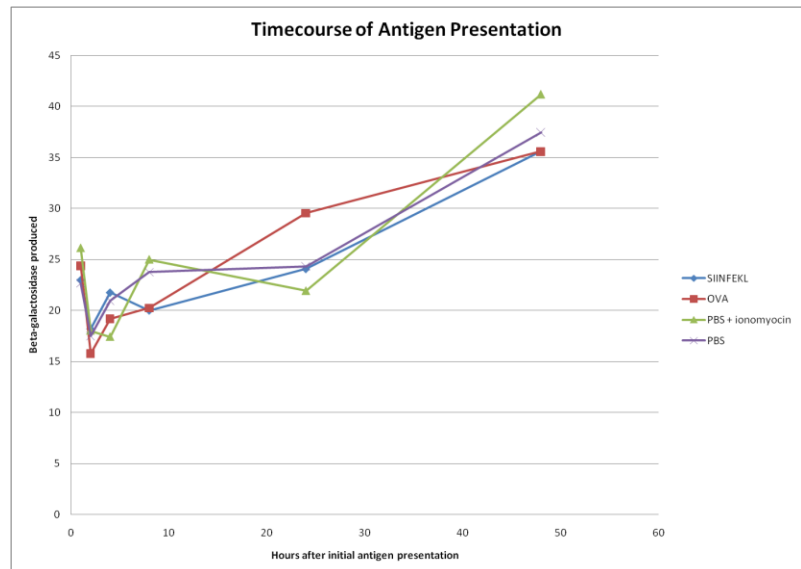
Since an increased response was not observed with the super-activation of splenocytes, a time course of antigen presentation was conducted to determine the stability of the presented peptides in the MHC I. The splenocytes were super-activated

with ionomycin for 48 hours before adding the antigen. The splenocytes were then incubated with the antigen for a given amount of time to allow for presentation. B3Z cells were then added to the splenocytes to become activated by any SIINFEKL that was able to be presented by the splenocytes.

Both OVA and SIINFEKL were assayed for antigen presentation over time. The controls of PBS or PBS and ionomycin were also assayed at each time point. The antigens were allowed to incubate with the splenocytes for 1, 2, 4, 8, 24, or 48 hrs before adding the B3Z cells. The time allowed for antigen presentation did not appear to affect the activation of the B3Z cells. At no time point was there significant β -gal production by either antigen above the base lines of PBS and PBS with ionomycin (Figure 5).

Figure 5: Time course of Antigen Presentation.

Splenocytes were super-activated with ionomycin for 48 hrs before adding antigens. The antigens OVA and SIINFEKL incubated with the splenocytes for the indicated amount of time before adding B3Z cells and incubating for an additional 24 hrs to allow for activation. The activation of B3Z cells is reported as the amount of β -gal produced.



Discussion:

In an attempt to increase antigen presentation, splenocytes were super-activated with ionomycin, which acts to increase calcium influx and activate cytokine release. The super-activation however did not increase the presentation of any of the four antigens assayed. The only antigen with β -gal production significantly above the PBS plus ionomycin control was SIINFELK. This only occurred at two of the lower concentrations. The antigen EVIrGM, which was able to elicit the most β -gal production using the RPMI method, was not able to produce a significant amount of β -gal.

This raised the concern of how long the peptide will stay presented on the cell surface, since the antigens were incubated with the splenocytes for 48 hrs before the B3Z cells were added. If the peptide/MHC I was not able to stay presented a substantial period of time, the splenocytes would not be able to activate the B3Z cells, as was observed. Hosken *et al.* (1989) showed that presentation of OVA peptides took as little as 45-60 minutes to be presented on an antigen presenting cells surface. Ludewig *et al.* (2001) demonstrated that while a specific antigen was able to be presented quickly in the MHC I and induce a cytotoxic T cell response, the presented peptides lasted for less than one day.

To investigate this issue, a time course of antigen presentation was done. Splenocytes were first super-activated with ionomycin and incubated with antigen for set amounts of time before adding the B3Z cells. There was no difference in B3Z cell activation for any of the time points. The β -gal production was never significantly greater than that of the PBS or PBS plus ionomycin controls. This suggests that either

the splenocytes are not able to present the SIINFEKL peptide at all, or the B3Z cells are not responding to the presentation. Even when the peptide SIINFEKL itself was presented to antigen presenting cells, the B3Z cells were not activated. While OVA would have to be processed by the cell into the SIINFEKL peptide, it should be much easier for the cell to present the peptide, since it should not need to be processed. This however did not appear to be the case.

CHAPTER 3: ASSAYS FOR B3Z FUNCTIONALITY

Introduction:

The results of the previous experiments have suggested that the B3Z cells are unresponsive to peptides presented in the MHC I of murine splenocytes. This could be a result of the splenocytes being unable to process and present the antigens. However, not even the SIINFEKL peptide was able to induce β -gal production by the B3Z cells. Instead, it was hypothesized that the B3Z cells were not able to be activated. In order to determine the functionality of the B3Z cells, several assays tested for T cell receptor (TCR) presence and activity, as well as the presence of the LacZ gene.

To determine if the B3Z cells were able to produce β -gal, they were stimulated with a combination of Phorbol 12-Myristate 13-Acetate (PMA) and ionomycin. The B3Z cells are constructed so that the LacZ gene, which codes for β -gal, is under the control of the nuclear factor of activated T cells (NF-AT) element of the IL-2 gene. When the T cell receptor (TCR) gets activated by binding the specific MHC/peptide, the receptor sends a signal through calcium dependent pathways including protein kinase C (PKC) and calmodulin to activate NF-AT, which then stimulates the transcription of IL-2. Since β -gal is under control of the IL-2 enhancer, it will also get transcribed upon TCR activation (Karttunen & Shastri, 1991). The PMA and ionomycin are able to enter the cells and simulate TCR activity. The combination of the two has shown to increase calcium influx and activate PKC and calcium-calmodulin pathways (Chatila *et al.*, 1989).

They have also been shown to directly activate β -gal production in B3Z cells (Kartunnen & Shastri, 1991).

If the B3Z cells were able to produce β -gal, the next step would be to look for TCR activation. In order to assay this, splenocytes were fixed with glutaraldehyde to prevent protein processing. When cells are fixed, the SIINFEKL peptide is still able to be presented by MHC I molecules already on the surface of the antigen presenting cells. Hosken *et al.*, (1989) showed that OVA peptides could be presented on the cell surface without internalization. These peptide/MHC I complexes were then able activate a cytotoxic T lymphocyte response (Hosken *et al.*, 1989). By mixing the splenocytes with SIINFEKL and fixing the cells, SIINFEKL should be presented in the MHC I on the surface of the cells and should be able to activate the TCR on B3Z cells. Therefore if the TCR is functional, it should cause β -gal production.

In another attempt to increase antigen presentation, and therefore activate the TCR of B3Z cells, splenocytes were super-activated with a much larger concentration of ionomycin. Ionomycin is able to cause production of the cytokines IL-2 (Chatila *et al.*, 1988) and interferon- γ (Jabrane-Ferrat *et al.*, 1999). The supplementary effect of ionomycin is to increase the number of MHC I molecules by increasing the production of interferon- γ . Interferon- γ has specifically been shown to up-regulate the transcription of the class I MHC heavy chain (Schroder *et al.*, 2004). For this reason, splenocytes were also super-activated specifically with interferon- γ .

In addition to up-regulating the MHC I, interferon- γ has been shown to increase the number of N-extended peptides formed by proteosomes in the cytosol. The

N-extension binds to a receptor on the ER membrane, allowing it to be transported into the ER. Once in the ER, the N-extension is cleaved off and the peptide is available to be combined with an MHC I (Rock *et al.*, 2004). Not only does interferon- γ increase the number of peptides transported into the ER by N-extension, but it has also been shown to specifically increase the number of SIINFEKL peptides produced from the processing of ovalbumin. In the presence of interferon- γ , proteosomes form immunoproteosomes which are able to increase the number of intact SIINFEKL peptides from 6% in normal proteosomes to 11% (Rock *et al.*, 2004).

Finally, in order to assay for the presence of the TCR on the B3Z cells surface, an immunofluorescence assay was conducted. Using new B3Z cells obtained from the University of California Berkeley, courtesy of Dr. Shastri, the TCR of the new B3Z cells were then compared to the TCR of B3Z cells used for all previous experiments. The “old” B3Z cells had been in culture for over a year. A decreased presence of the TCR could explain the lack of activation of the B3Z cells by the splenocytes presented with antigens containing the SIINFEKL peptide.

Methods:

Activating B3Z cells with PMA/ionomycin:

B3Z cells were activated with PMA and ionomycin as described previously. A serial dilution of PMA and ionomycin was added to B3Z cells. After a 4 hour incubation, the β -gal production by the B3Z cells was measured by luminescence using the Luminescent β -gal detection kit II (Clontech) and the GloRunner Luminometer. The

assay was conducted with B3Z cells grown in IMDM, cells grown in RPMI, and cells that were grown in RPMI but then had the media switched to IMDM.

Fixing splenocytes:

Splenocytes were isolated into AIM-V media as described previously. Antigens were then added to the splenocytes and incubated for 15 min. Half of the cells for each antigen type were fixed using glutaraldehyde. The cells were incubated for an additional hour before adding B3Z cells. The B3Z cells were incubated with the splenocytes for 4 hrs before determining the β -gal production using the Luminescent β -gal detection kit II (Clontech) and the GloRunner Luminometer.

Super-activating splenocytes with increased ionomycin concentration:

Splenocytes were isolated into AIM-V media as described previously. Serial dilutions of the antigens were added to the splenocytes with 5 μ M ionomycin. Other wells containing a serial dilution of OVA were treated with 70 μ M ionomycin. Controls were PBS, PBS plus 5 μ M ionomycin, and PBS plus 70 μ M ionomycin. After super-activating for 48 hrs, B3Z cells were added. After an additional 24 hr incubation, the β -gal production was determined using the Luminescent β -gal detection kit II (Clontech) and the GloRunner luminometer.

Super-activating splenocytes with interferon- γ :

Splenocytes were again isolated into AIM-V media as described previously. Antigens were plated at a constant concentration and a serial dilution of interferon- γ , starting with 200 μ M interferon- γ . SIINFEKL and OVA were both used at 10 μ M. A 200 μ M interferon- γ control alone was used. The splenocytes were incubated for 48 hrs before adding B3Z cells and incubating an additional 24 hrs. The β -gal production was then determined using the Luminescent β -gal detection kit II (Clontech) and the GloRunner luminometer.

Immunofluorescence of TCR on B3Z cells:

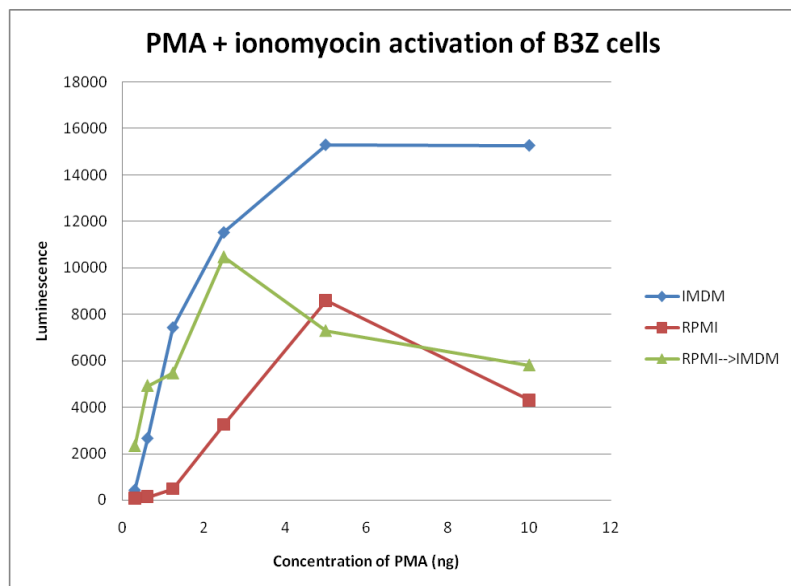
To assay for the presence of the T cell receptor (TCR) on the surface of the B3Z cells, an immunofluorescence assay was conducted. The primary antibody was a mouse antibody specific to the TCR. The secondary antibody was specific to mouse IgG and conjugated to biotin. Streptavidin-FITC was then used to detect the biotin. The FITC fluoresced green under the fluorescent microscope. The cells were stained with DAPI to visualize their nucleus, which fluoresced blue. New B3Z cells, obtained from the University of California Berkeley were compared to the B3Z cells used previously that had been in culture for over a year for the presence of the TCR.

Results:

To assay if the B3Z cells were still capable of producing β -gal, the cells were stimulated with a combination of PMA and ionomycin. Cells grown in the two types of

media used in the previous experiments, RPMI or IMDM, were compared. Cells grown in RPMI but switched to IMDM media two days prior were also assayed. The cells were able to produce β -gal as demonstrated by the large luminescence measured (Figure 6). All points were significantly greater than the PBS control. The PMA or ionomycin when added individually did not activate β -gal production (data not shown); only the combination of the two was able to stimulate β -gal production. The IMDM had a greater luminescence compared to the cells grown in RPMI, indicating more β -gal production.

Figure 6: PMA and Ionomycin activation of B3Z cells. B3Z cells grown in two different types of media were incubated with serial dilutions of PMA and ionomycin. The β -gal production was measured by luminescence. All points were significantly ($p \leq 0.05$) greater than the luminescence of the B3Z cells treated with only PBS. Ionomycin and PMA when added individually were not able to stimulate B-gal production (data not shown).



Splenocytes were then presented with either SIINFEKL or OVA and fixed with glutaraldehyde to prevent the cell from processing the antigen. The SIINFEKL should be able to combine with MHC I molecules on the splenocyte surface and subsequently activate the B3Z cells, if the B3Z cells have a functioning TCR. The fixed splenocytes were able to significantly activate B3Z cells at some antigen concentrations using both

the OVA and SIINFEKL compared to the control of splenocytes that were fixed without antigen (Figure 7). The cells presented with OVA that were not fixed also had some activation significantly greater than the control cells not fixed but treated with PBS. The cells presented with SIINFEKL did not.

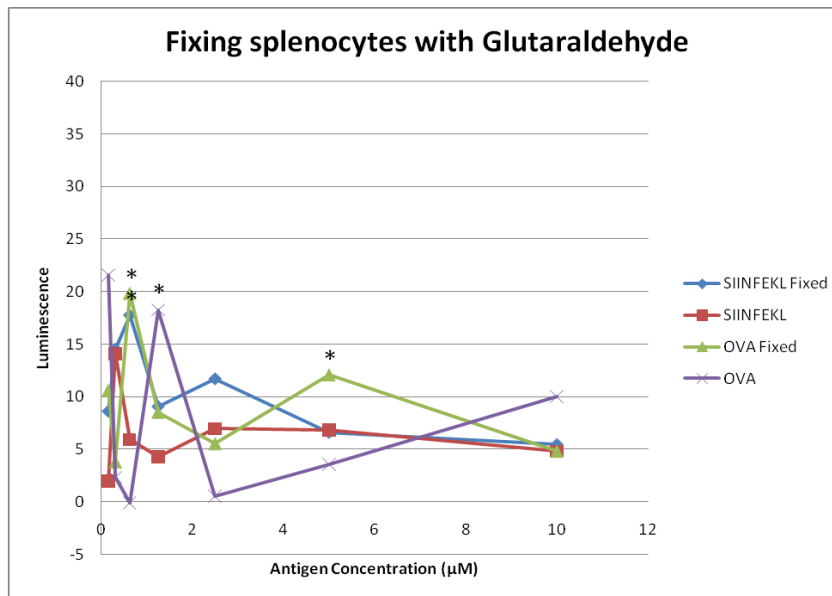


Figure 7: Fixing Splenocytes with Glutaraldehyde. Splenocytes were incubated with antigen for 15 min before fixing with glutaraldehyde. B3Z cells were added one hour later and allowed to incubate for 24 hrs. Significant ($p \leq 0.05$) luminescence above the PBS control or fixed cells with PBS control is indicated by the stars (*).

When splenocytes were super-activated with a large amount of ionomycin (70 μM), the cells were able to activate B3Z cells to produce $\beta\text{-gal}$, but in an antigen independent manner. When SIINFEKL and OVA were presented to the splenocytes and super-activated with 5 μM ionomycin as before, there was no significant $\beta\text{-gal}$ production above the PBS plus ionomycin control (labeled as PBS + ionomycin in Figure 8). The OVA plus 70 μM ionomycin stimulated significant $\beta\text{-gal}$ production compared to the 5 μM ionomycin and OVA. However the $\beta\text{-gal}$ production was

significantly less than the 70 μ M ionomycin plus PBS. It was therefore concluded that the 70 μ M ionomycin was itself causing the activation rather than the OVA (Figure 8).

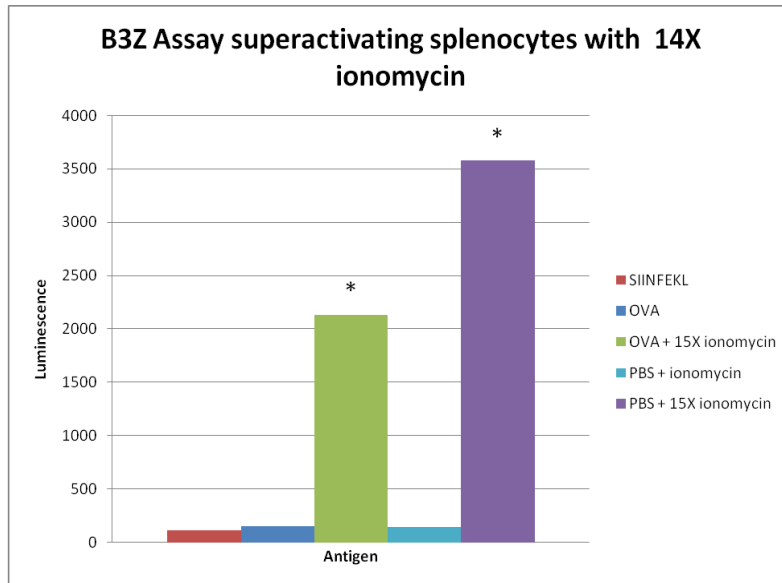
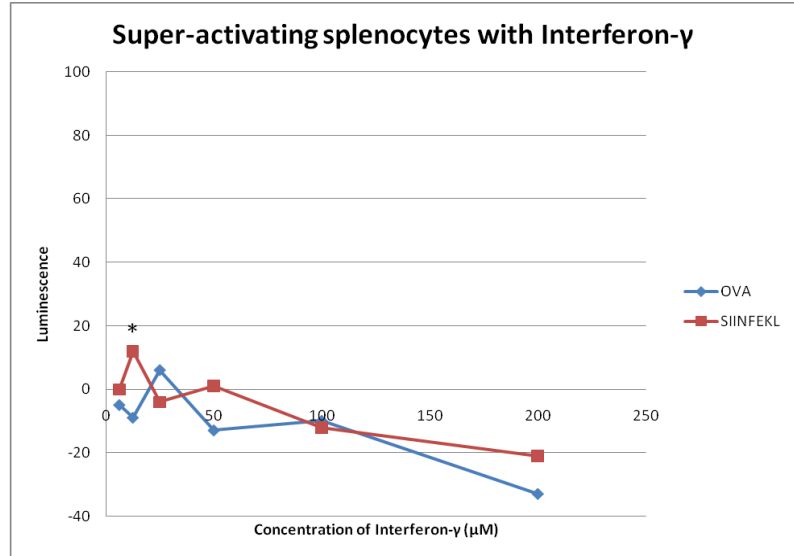


Figure 8: Super-activating splenocytes with increased ionomycin. Splenocytes were super-activated with 14 times the ionomycin as used in previous super-activations. The antigens were all added to 10 μ M and compared to PBS alone. The stars (*) indicate luminescence significantly above the control of PBS alone and 5 μ M ionomycin ($p < 0.05$).

To avoid the antigen independent activation of the B3Z cells, splenocytes were super-activated with interferon- γ , which still increases the number of MHC I molecules on the antigen presenting cells. The splenocytes were incubated with a constant concentration of antigen but a serial dilution of interferon- γ to determine what concentration would have the optimal effect for super-activation. The super-activation with interferon- γ did not have any effect on the ability of the splenocytes to activate the B3Z cells. There was only one concentration of SIINFEKL with significant β -gal production compared to the controls, while the remaining concentrations of interferon- γ did not differ from the controls (Figure 9).

Figure 9: Super-activating splenocytes with Interferon- γ . Splenocytes were super-activated with decreasing concentrations of interferon- γ and set concentration of antigen to determine the optimal amount of interferon- γ to super-activate splenocytes. The antigens OVA and EVI were at a concentration of 10 μ M. Luminescence was then compared to the PBS control. Stars (*) indicated significant readings above the control ($p \leq 0.05$).



An immunofluorescence study of the B3Z cells was conducted to test for the presence of the TCR. New B3Z cells were obtained from UC Berkeley, and compared to the B3Z cells that had been used in all previous experiments and had been in culture for over a year. The TCR was detected using an antibody conjugated to FITC which fluoresced green. The cells were stained with DAPI which fluoresced blue. As can be seen in Figure 10, the new B3Z cells had a much higher density of green staining, indicating an increased number of TCR compared to the old B3Z cells.

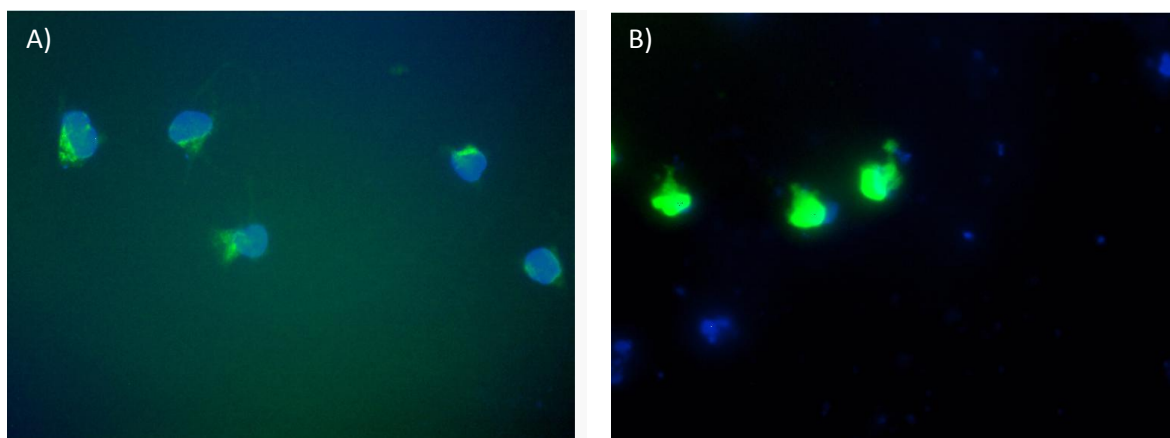


Figure 10: Immunofluorescence of B3Z cells. The TCR of B3Z cells was detected using a mouse antibody specific to the TCR. The secondary antibody was specific to mouse Ig and conjugated to biotin. The biotin was then fluoresced green using streptavidin-FITC. The cells were stained with DAPI and fluoresced blue. Both pictures are magnified at 40X on a fluorescent microscope. (A) shows the old cells. (B) shows the new cells from UC Berkeley.

Discussion:

Previous experiments during this project had suggested that the B3Z cells used were not able to be adequately activated. To determine if the B3Z cells were still capable of producing β -gal, they were activated with ionomycin and PMA. The ionomycin and PMA act together to increase calcium signaling, leading to the transcription of the LacZ gene of the B3Z cell. The B3Z cells were able to make β -gal, and did so in correlation to the amount of ionomycin and PMA added. This result indicates the LacZ gene is intact and functional. The B3Z cells grown in IMDM media showed a greater luminescence and better correlation to PMA and ionomycin concentration compared to those grown in RPMI.

Since the B3Z cells were able to transcribe and produce β -galactosidase, it was next assayed whether the TCR was able to recognize SIINFEKL in the MHC I. This was

determined without the splenocytes having to process the antigens by presenting the SIINFEKL to the splenocytes and then fixing them with glutaraldehyde. The SIINFEKL can bind to empty MHC I molecules on the surface of the antigen presenting cell. If the TCR of the B3Z cells are functional, the B3Z cell should be activated by the fixed splenocytes.

Some concentrations of SIINFEKL and OVA were able to stimulate β -gal production in B3Z cells exposed to the fixed splenocytes that was greater than the β -gal production of the B3Z cells exposed to the fixed splenocytes without antigen control. However, there was no correlation between the amount of antigen presented and the amount of β -gal produced. OVA should not be able to bind to MHC I on the outside of the splenocyte because only peptides 8-12 amino acids long can fit in the MHC I complex (Hosken *et al.*, 1989). OVA would need to be processed by the cell in order to produce the necessary peptides, and therefore served as a negative control. The observation that OVA was able to give readings significantly above control and at about the same value as the SIINFEKL that gave significant readings, suggests that B3Z cells were not actually being activated by the antigen in the MHC I.

Presentation of OVA by splenocytes that were not fixed also gave significant readings above the PBS control. While the readings were statistically significant, it was interesting to note that they did not differ from the OVA readings obtained from the fixed cells. The unfixed cells presented with SIINFEKL did not significantly stimulate β -gal production. It could be assumed that the fixed cells, while able to bind and present the SIINFEKL peptide in MHC I molecules present on the cell surface, would not be expected to present a significant amount of antigen when compared to cells that were

not fixed and allowed to process the antigen. These results support the conclusion that the “old” B3Z cells are not responding to the peptide presented in the MHC I.

In an attempt to get the splenocytes to present more antigen peptides and therefore possibly activate the B3Z cells, splenocytes were super-activated with a large (70 μ M) concentration of ionomycin. While the lower concentration (5 μ M) of ionomycin was not able to cause β -gal production in cells presented with OVA, the higher concentration (70 μ M) did. When this higher concentration of ionomycin was added to cells with PBS instead of antigen however, an even larger response was seen. This demonstrated that it was the ionomycin, and not the OVA, that was activating the B3Z cells.

The ionomycin must have been activating the splenocytes, which then were able to activate the B3Z cells, since the ionomycin itself was washed off before the B3Z cells were added when the media was changed from AIM-V to IMDM. The activation of the B3Z cells was in an antigen independent manner, since the OVA did not cause an increased response when mixed with the high concentration of ionomycin compared to the high concentration of ionomycin alone.

In order to avoid the T cell activation by ionomycin affecting the splenocytes, but still be able to get the favorable effect of enhanced MHC I production by the splenocytes, interferon- γ was used to super-activate the cells. Not only should it increase the number of MHCs, but it has been shown to increase the amount of intact SIINFEKL peptides produced by the proteosomes. However, when interferon- γ was added at differing concentrations to the splenocytes, it had no effect on the β -gal

production by the B3Z cells. We concluded this could be attributed to the lack of a functioning TCR.

To assay if the TCR was still present on the surface of the B3Z cells, an immunofluorescence assay was done. At this time new B3Z cells had been obtained from UC Berkeley. The number of TCRs present on the new B3Z cells could then be compared to the B3Z cells that had been used for the previous experiments. When the cells were stained with antibodies specific to the TCR and then detected with FITC, there appeared to be a much larger number of receptors on the new cells compared to the old. It appeared that the old cells had lost some of their TCRs, and this may explain the low levels of activation by SIINFEKL presented in the MHC I of splenocytes. The previous assays were then repeated using the new cells with the larger number of TCRs.

CHAPTER 4: ENZYME-LINKED IMMUNOSORBANT ASSAY OF SPLENOCYTES

Introduction:

The goal of this project was to increase the cross-presentation of the tumor antigen HER2 by antigen presenting cells. In order to measure the amount of cross-presentation, a modified version of the HER2 protein containing the SIINFEKL peptide was used. When presented by antigen presenting cells, it should activate the B3Z cell line specific to the SIINFEKL peptide presented in the MHC I. Activation of the B3Z cells had not been observed through the presentation of SIINFEKL from any of the antigens presented to the splenocytes, or by the SIINFEKL peptide itself. In attempt to measure the peptide presentation without using the B3Z cells, an enzyme linked immunosorbant assay (ELISA) was conducted. This would provide a way to quantify antigen presentation without relying on the activation B3Z cells.

Methods:

Splenocytes were isolated into serum free AIM-V media as described previously and super-activated with antigen and 5 μ M ionomycin for 48 hrs. OVA, EVI and SIINFEKL were all used in serial dilutions. The splenocytes were assayed by ELISA as described previously. Briefly, the splenocytes were fixed with formaldehyde and the wells were blocked with goat serum. The antibody 25-D1.16 conjugated to biotin was incubated with the cells for one hour. The antibody 25-D1.16 is specific to SIINFEKL presented in the MHC I. The avidin-horse radish peroxidase (HRP) conjugated molecule was added

and incubated an additional hour. The HRP was detected using an ELISA specific HRP chemiluminescent detection kit (Pierce). Controls consisted of splenocytes with PBS instead of antigen, splenocytes in media without anything added, and wells without cells that were fixed, blocked and washed the same as the wells with cells.

Results:

The ELISA of SIINFEKL in the MHC I using splenocytes was meant to provide a method of measuring antigen presentation without using the B3Z cells. The amount of SIINFEKL presented in the MHC I was not able to be detected above the luminescence of the PBS plus ionomycin control. None of the antigens (OVA, SIINFEKL, or EVI) stimulated significant luminescence above the cells only control (Figure 10). The presentation of SIINFEKL in the MHC I could not be detected using the antibodies and HRP luminescent kit.

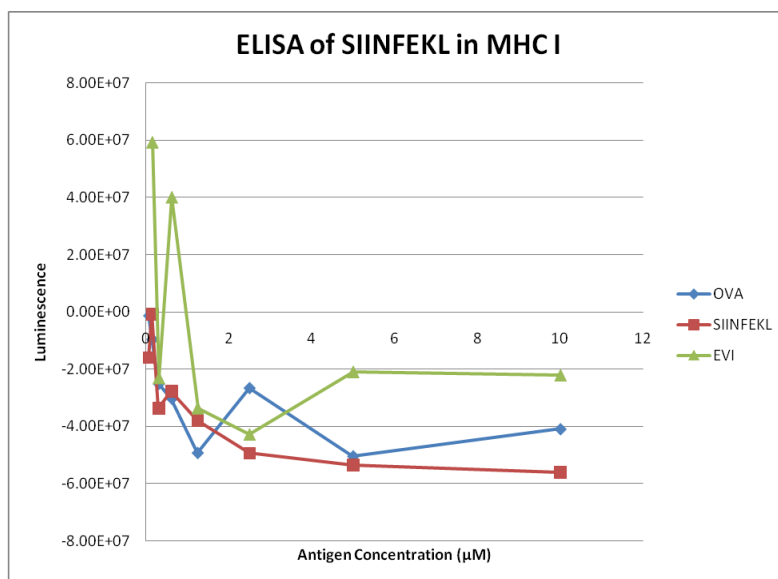
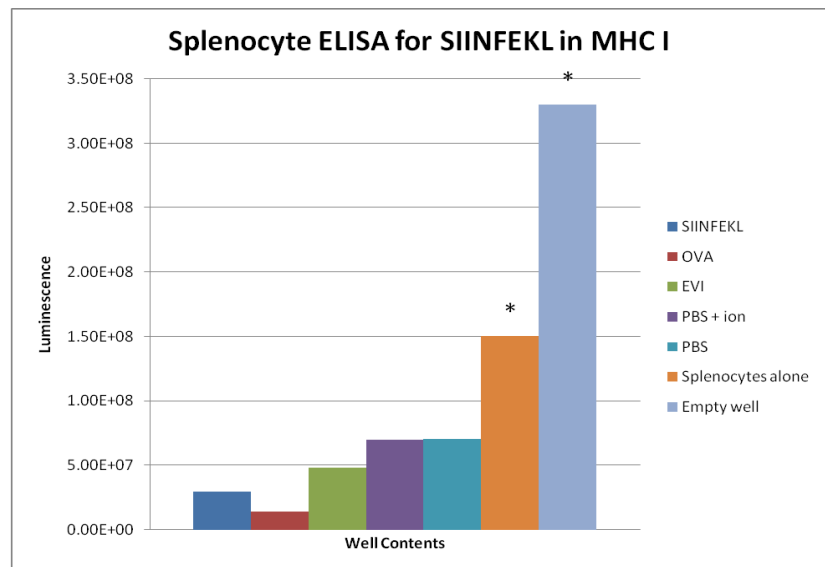


Figure 11: ELISA of SIINEKL in the MHC I. Splenocytes were super-activated with ionomycin and a serial dilution of antigen. SIINFEKL/MHC I complexes were then detected using a biotin conjugated antibody. Avidin-HRP was used to detect the biotin. HRP was detected using a chemiluminescent kit. Luminescence readings were not significantly greater than controls with PBS and ionomycin for any concentration of antigen.

When the highest antigen concentration for each antigen was compared to the cells only control or to empty wells that were treated the same, it was observed that the cells alone and the empty wells gave the largest luminescent readings. Both were significantly greater than the PBS plus ionomycin control (Figure 12).

Figure 12: Comparing Controls of splenocyte ELISA. The highest concentration of each antigen in Figure 11 is shown compared to the controls and empty wells. The stars (*) indicate luminescent readings significantly above that of the cells with PBS and ionomycin ($p < 0.05$).



Discussion:

In order to detect antigen presentation without using the cytotoxic T cell line B3Z, an ELISA for SIINFEKL was conducted. An antibody specific to the peptide SIINFEKL presented in the MHC I was used to determine the amount of antigen presentation from serial dilutions of the antigens OVA, EVI and SIINFEKL. No significant presentation could be detected using this method. While the EVI gave some large luminescent readings at the smaller concentrations, they were not significantly greater than the PBS control. The OVA and SIINFEKL gave no readings greater than the PBS control.

This suggests that either the SIINFEKL peptide is not being presented by the splenocytes, or this method was not an accurate way to detect antigen presentation. If there was a lot of non-specific binding by the antibody or the avidin molecule, a significant reading of SIINFEKL presentation may not be detectable. This seemed to be the case, since the luminescent readings of cells alone or the wells that were processed the same as the wells with cells gave luminescent readings significantly above the PBS control. The wells that were blocked with goat serum, washed, and incubated with the antibody and avidin in the same process as the wells with splenocytes showed the highest luminescence of all the wells. They were significantly greater than the PBS with splenocytes control. This suggests that there may be non-specific binding to the wells that was not corrected by blocking with the goat serum.

Considering the cells in media alone, with no PBS or ionomycin added, resulted in a luminescent reading that was significantly higher than PBS or PBS plus ionomycin controls suggests there was non-specific binding by either the antibody or the avidin. If this is the case, the ELISA, in our hands, does not seem to be an accurate measure of antigen presentation. The new B3Z cells from UC Berkeley were next tested for their ability to be activated by splenocytes, since it was demonstrated that they have more TCRs than the old B3Z cells used in previous experiments (Chapter 3).

CHAPTER 5: ASSAYS USING NEW B3Z CELLS

Introduction

After demonstrating that the B3Z cells received from UC Berkeley contained more TCRs than the previously used B3Z cells (Chapter 3), the B3Z assays were repeated using the new cells (hereafter referred to as new B3Z cells). With more TCRs available, the activation of the B3Z cells should be assayable if the SIINFEKL peptide is presented by the splenocytes. The presentation of the antigens was assayed using the RPMI and both of the super-activation methods to find which method gave the greatest activation of B3Z cells as determined by β -gal production. The cells were also fixed with glutaraldehyde to determine if the SIINFEKL peptide was entering the cell and undergoing the presentation process, or just binding to MHC I molecules on the outside of the antigen presenting cells.

Methods

B3Z assay in RPMI

A B3Z assay was set up using the RPMI method described previously. Fifty microliters of splenocytes at a concentration of 2.6×10^6 cells/mL and B3Z cells at 1.4×10^6 cells/mL in complete RPMI were mixed with serial dilutions of the antigens SIINFEKL, OVA and EVI starting with 10 μ M. The control was splenocytes and B3Z cells with PBS (no antigen). The plate was incubated for 24 hrs at 37 °C and 5% CO₂ before

determining the β -gal production using the Luminescent β -gal Detection kit II (Clontech) and a Modulus microplate reader (Turner Biosystems).

Super-activating Splenocytes with ionomycin

A B3Z assay was set up to super-activate 100 μ L of splenocytes at a concentration of 1.3×10^6 cell/mL for 48 hrs in AIM-V media with 5 μ M ionomycin and a serial dilution of antigen as described previously. The dilutions were started with 10 μ M SIINFEKL, OVA or EVI. Controls were cells treated with PBS alone or PBS plus ionomycin. The media was switched to IMDM and 100 μ L of B3Z cells at a concentration of 7.0×10^5 cell/mL were added. The plate was incubated for an additional 24 hrs before determining the β -gal production using the Luminescent β -gal Detection Kit II (Clontech) and the Modulus microplate reader.

Super-activating splenocytes with interferon- γ

Splenocytes were super-activated as described previously in AIM-V media with either 10 μ M SIINFEKL or OVA and a serial dilution of interferon- γ , starting with 200 μ M. A serial dilution of interferon- γ without antigen was used to determine if the interferon- γ itself was causing activation at each concentration. After 48 hrs, B3Z cells in IMDM were added and the plate was incubated for 24 hrs more. The β -gal production was then measured using the Luminescent β -gal Detection Kit II (Clontech) and a Modulus microplate reader.

Fixing splenocytes with glutaraldehyde

Splenocytes were incubated with antigen for 15 min. and then fixed with glutaraldehyde as described previously. The antigens were SIINFEKL and OVA serially diluted starting with 10 μ M. The cells were then incubated for one hour before adding the B3Z cells and incubating an additional 24 hrs. The β -gal production was then measured using the Luminescent β -gal Detection Kit II (Clontech) and a Modulus microplate reader.

Results:

The new B3Z cells were first assayed using the RPMI method. Splenocytes and B3Z cells were mixed together with serial dilutions of antigen and incubated for 24 hrs. The SIINFEKL caused β -gal production greater than the PBS control (Figure 13). Not only did it cause significant β -gal production at every concentration of SIINFEKL, but it was also correlated to the amount of antigen presented. The OVA and EVI however did not give significant β -gal production compared to the control.

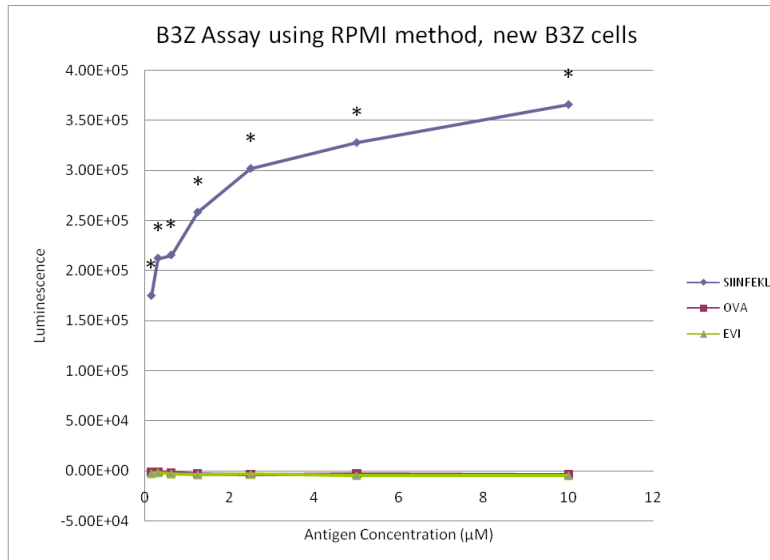


Figure 13: B3Z assay using RPMI method, new B3Z cells. Splenocytes and B3Z cells were mixed with serial dilutions of SIINFEKL, OVA or EVI and incubated for 24 hrs. The β -gal production was then measured as a luminescent reading. All of the SIINFEKL readings were all significantly above the control of PBS, as indicated by the stars (*), while the OVA and EVI were not significant ($p \leq 0.05$).

The splenocytes were then super-activated with ionomycin to determine if the presentation of SIINFEKL from the EVI and OVA proteins could be elicited, or if the β -gal production from the antigen SIINFEKL could be increased. The SIINFEKL again produced significant β -gal production by the B3Z cells when compared to the control of PBS and ionomycin (Figure 14). The OVA and EVI however did not cause significant β -gal production (Figure 14).

When the SIINFEKL β -gal production by the super-activation method was compared to the RPMI method, it had significantly more β -gal production at all concentrations except for the 5 μ M (Figure 15). A β -gal standard was used to calculate the amount of β -gal produced by the cells in each case. The luminescent values between the two assays could then be compared. While the super-activation method was not able to increase the β -gal production by EVI or OVA presentation, it caused significant increase in the presentation of the SIINFEKL peptide compared to the RPMI method.

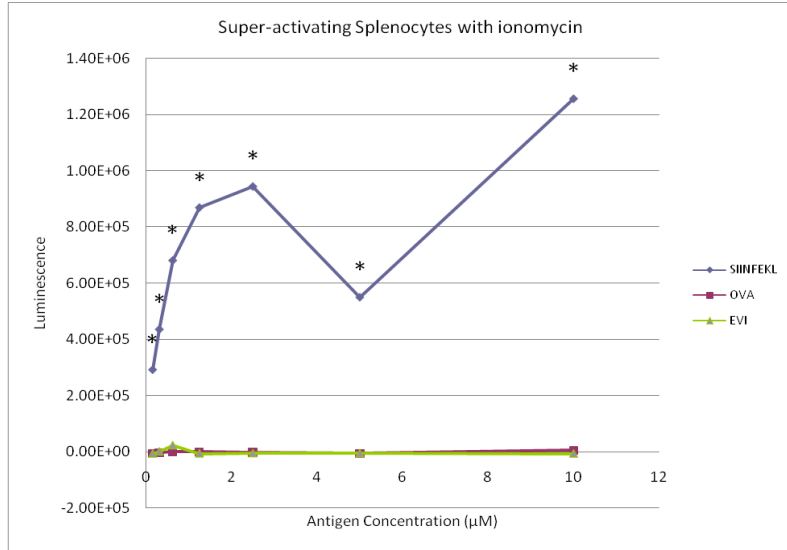
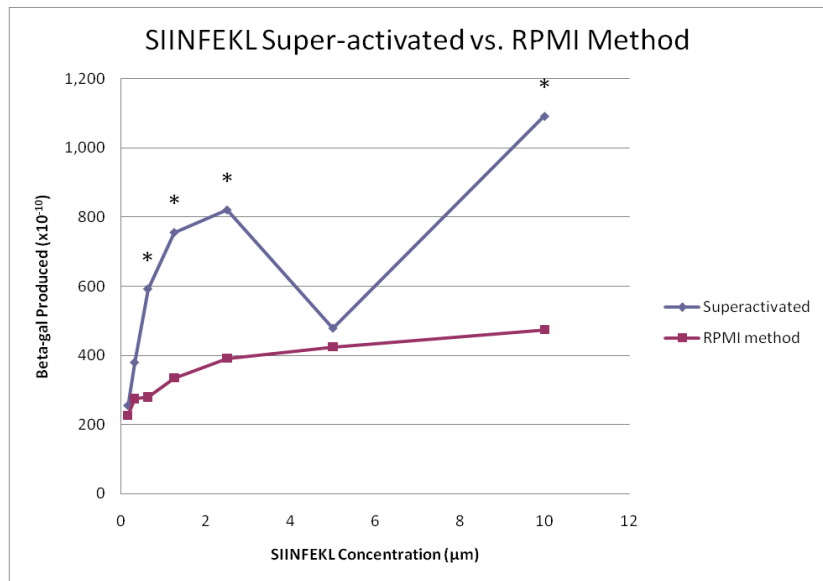


Figure 14: Super-activating splenocytes with ionomycin. Splenocytes were incubated with a serial dilution of the antigens SIINFEKL, OVA or EVI and 5 μM ionomycin for 48 hrs before adding B3Z cells. The β-gal production was then measured by luminescence. The SIINFEKL readings were significantly ($p < 0.05$) greater than the control of PBS and ionomycin.

Figure 15: Comparison of SIINFEL presentation by the RPMI method to super-activation. The β-gal produced by SIINFEL presentation using the RPMI method is shown compared to the the β-gal produced when SIINFEL was presented with super-activation of the splenocytes. The super-activation method gave significantly higher β-gal production than the RPMI method, as indicated by the stars (*) ($p \leq 0.05$).



The splenocytes were super-activated with interferon-γ as done previously. A serial dilution of interferon-γ with either 10 μM SIINFEKL or OVA at each concentration of interferon-γ. The SIINFEKL showed significantly higher β-gal production when

compared to the PBS control (Figure 16). The interferon- γ dilution itself, without antigen, was significantly higher than the PBS control at one concentration. When SIINFEKL was compared to the interferon- γ at each concentration, SIINFEKL was significantly higher than the interferon- γ alone. The OVA in combination with interferon- γ did not have any significant β -gal production compared to the PBS control.

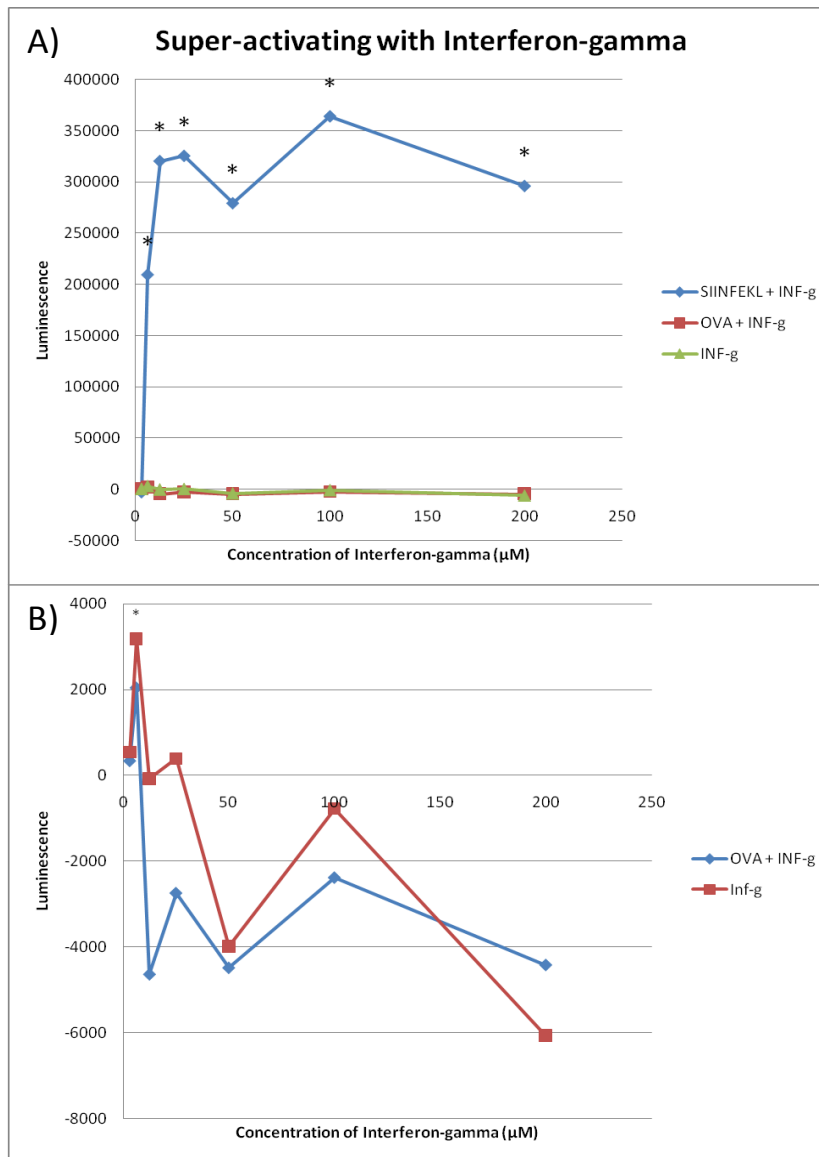
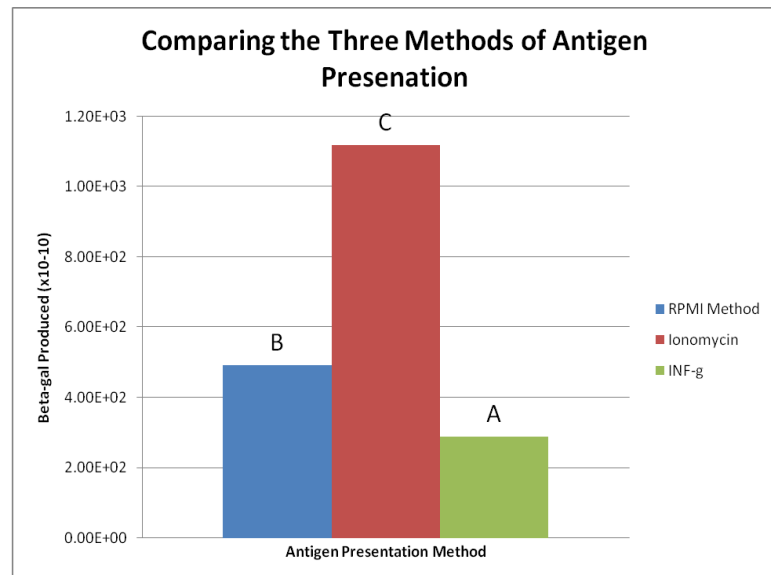


Figure 16: Super-activating splenocytes with interferon- γ . Splenocytes were activated with a set amount of antigen, either 10 μ M SIINFEKL or OVA, and a serial dilution of interferon- γ for 48 hrs. The B-gal produced was then compared to the PBS control. (A) SIINFEKL produced significantly more β -gal than the PBS control. The SIINFEKL + interferon- γ was also compared to the interferon- γ alone at each concentration for β -gal production and was found to be significantly greater ($p \leq 0.05$) as indicated by the stars (*). (B) The interferon- γ dilution without antigen did not differ from the PBS control except at 6.25 μ M as indicated by the star.

Since the SIINFEKL was presented to the splenocytes using all three antigen presentation methods at 10 μ M, the β -gal production as a result of 10 μ M SIINFEKL presentation was compared between the three methods (Figure 17). The comparison of all three methods showed they were significantly different in their β -gal production ($p \leq 0.05$). The super-activation with ionomycin gave the most production, while the interferon- γ super-activation gave the least.

Figure 17: Comparing the three methods of antigen presentation. The β -gal production as a result of presentation of 10 μ M SIINFEKL to splenocytes was calculated for each presentation method. The RPMI method produced significantly more β -gal than the Interferon- γ method and the ionomycin method produced significantly more β -gal than the RPMI method. Bars with different letters are significantly different ($p \leq 0.05$).



To assay if the splenocytes were processing the antigen, or if the SIINFEKL peptide was able to bind to MHC I on the outside of the cell, the splenocytes were fixed with glutaraldehyde. The splenocytes were presented with SIINFEKL or OVA for 15 min and then half of the splenocytes were fixed. The β -gal production of the B3Z cells mixed with the fixed splenocytes was compared to the fixed PBS control, while the non-fixed cells were compared to the PBS control with non-fixed cells.

The fixed cells that were presented with SIINFEKL did not cause significant β -gal production by the B3Z cells compared to the fixed control cells, with one exception (Figure 18B). The cells presented with SIINFEKL that were not fixed caused significantly greater β -gal production at all concentrations when compared to the non-fixed PBS control. When compared to the fixed cells presented with SIINFEKL, the non-fixed SIINFEKL presented cells also produced significantly greater β -gal at all concentrations of SIINFEKL (Figure 18A).

The fixed cells presented with OVA did not have significant β -gal production when compared to the control cells that were fixed and presented with PBS. The non-fixed, OVA presented cells did not differ from the PBS control except at two low concentrations (Figure 18B).

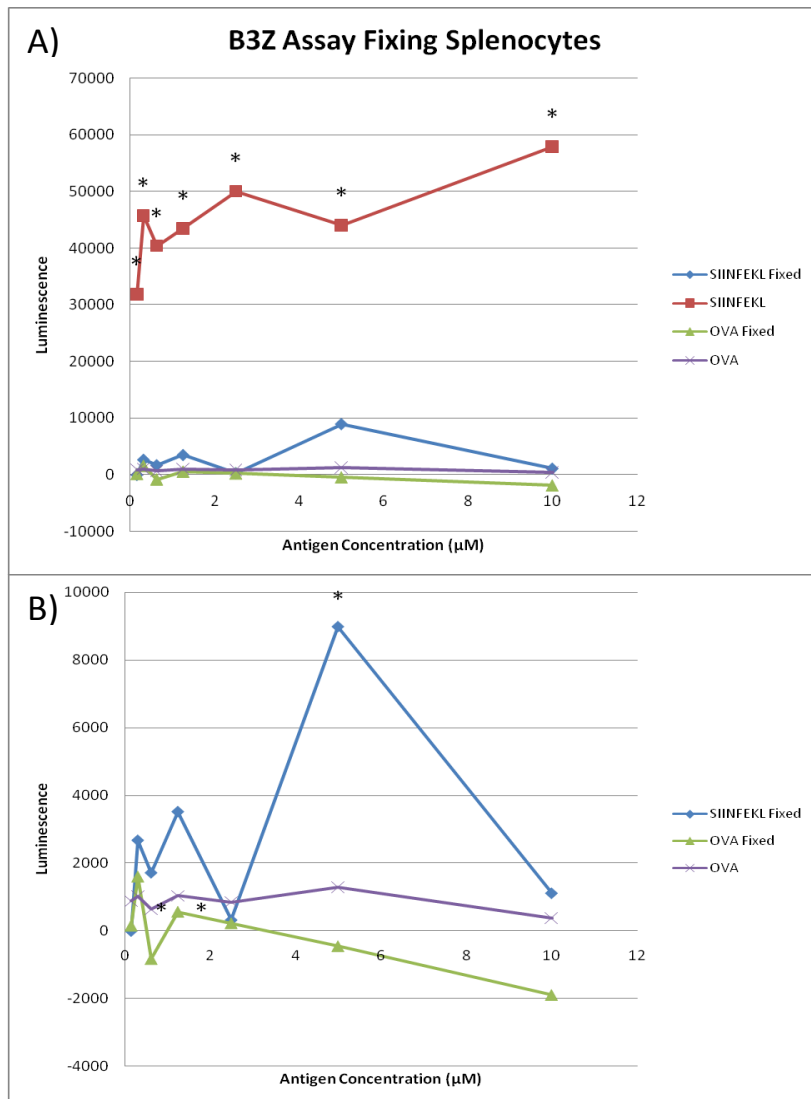


Figure 18: Effect of Fixing Splenocytes with Glutaraldehyde.

Splenocytes were presented with serial dilution of the antigens SIINFEKL or OVA for 15 min before fixing half the cells with glutaraldehyde. The cells were incubated for another hour before B3Z cells were added and then incubated an additional 24 hrs. (A) The SIINFEKL fixed was compared to the SIINFEKL presented to non-fixed cells and was found to be significantly lower at all concentrations. Fixed and non-fixed OVA did not differ. (B) The fixed cells treated with antigen were compared to fixed cells with PBS. Non-fixed cells were compared to non-fixed cells with PBS. Significant differences are indicated by stars (*) ($p \leq 0.05$).

Discussion:

The new B3Z cells received from UC Berkeley were assayed for their ability to be activated by the presentation of SIINFEKL from the antigens EVI, OVA and the peptide SIINFEKL itself. Since the new B3Z cells had been shown to have a larger number of TCRs than the old cells, they should be able to recognize antigen presented by the antigen presenting cells more robustly. The new B3Z cells were able to produce

significant β -gal production when the splenocytes were presented with SIINFEKL peptide using any of the three activation methods. In all three cases the response to SIINFEKL corresponded to the SIINFEKL concentration. This demonstrated that the B3Z cells were responding to a difference in presentation of the SIINFEKL peptide by becoming more activated with an increase in antigen concentration and therefore an increase in antigen presentation.

The SIINFEKL was able to cause significantly more activation when presented using the super-activation with ionomycin method then with the RPMI method. It is suspected that the increased MHC I production due to ionomycin's effects on the antigen presenting cells caused more presentation of the peptide. The B3Z cells were also able to respond to a difference in presentation of the SIINFEKL peptide by varying the concentrations of interferon- γ . The higher the concentration of interferon- γ used, the more SIINFEKL peptide was presented and therefore more activation of B3Z cells was observed through β -gal production and detected by luminescence.

While all three methods showed significant β -gal production when the cells were presented with SIINFEKL, the super-activation with ionomycin caused the most β -gal production. Since all three assays were done with 10 μ M SIINFEKL, the β -gal production resulting from presentation of 10 μ M SIINFEKL was compared between each method. The super-activation with ionomycin gave the largest production of β -gal, with the next largest from the RPMI method, and the smallest from the super-activation with interferon- γ . Even though interferon- γ has the advantage of making the antigen

presenting cells produce more MHC I molecules, the added effect of other pathways activated by ionomycin must also aid in increasing the presentation of SIINFEKL.

While the SIINFEKL peptide was able to be presented by the antigen presenting cells, the EVI and OVA proteins did not seem to be processed by APCs into the necessary SIINFEKL peptide for presentation by the splenocytes. The EVI and OVA did not give significant luminescent readings compared to controls using either the RPMI or the super-activating methods. While super-activating the splenocytes allowed for more SIINFEKL presentation, neither the EVI nor the OVA responded appropriately. This is an important observation when considering the optimal composition of a tumor vaccine. It suggests that a specific peptide ready to be directly combined with an MHC I would be able to mount a larger T cell response than a whole protein that needs to be processed by the cell. It implies that the most efficient and effective vaccine to elicit a cytotoxic T cell response is a specific antigenic peptide. A more detailed study on the peptides formed when HER2 is cleaved by proteosomes in the cell and which of these peptides are most efficiently cross-presented by antigen presenting cells resulting in the activation of cytotoxic T cells may lead to a new agent for vaccine formation.

To determine if the antigen presenting cells were actually internalizing the SIINFEKL peptide and cross-presenting it to the surface of the cell in an MHC I, the splenocytes were presented with SIINFEKL and OVA but then fixed to prevent the cells from further processing the antigen. If the SIINFEKL was simply combining with MHC I molecules already on the surface of the cell, then fixing the splenocytes should not affect the β -gal production from the B3Z cells in response to the presented SIINFEKL.

When the cells were fixed, the SIINFEKL presentation was significantly lower than when the cells were not fixed. This demonstrated that cross-presentation was occurring.

One concentration of SIINFEKL in the cells that were fixed was able to cause significant production of β -gal when compared to the control of the fixed cells presented with PBS. This suggests that it is possible for the SIINFEKL to combine with MHC I already on the surface of the splenocytes as described by Hosken *et al.* (1989). As expected, the OVA did not cause significant β -gal production when the splenocytes were fixed. The OVA protein is too large to fit in the MHC I already on the cell surface and therefore needs to be processed by the cell, so it should not be able to cause β -gal production when the cells are fixed, as observed.

Since the SIINFEKL was able to cause activation of the new B3Z cells as detected by β -gal production, it shows that the model is able to detect cross-presentation despite the negative outcomes of previous experiments. Because this was the case, the EVI protein was next conjugated to mannan to assay the original hypothesis that glycosylation would increase cross-presentation.

CHAPTER 6: GLYCOSYLATION OF EVI AND SIINFEKL

Introduction

Glycosylation of proteins has been demonstrated to not only increase antigen presentation (Tan *et al.*, 1997), but also increase cross-presentation of the antigen (Apostolopoulos *et al.*, 1995) through interaction with the mannose receptor (Burgdorf *et al.*, 2007). In an attempt to increase the cross-presentation of the tumor antigen HER2, the EVI variation of HER2 was glycosylated by conjugation to oxidized mannan according to Apostolopoulos *et al.* (1995). It was then assayed for its ability to be presented in the MHC I and cause activation of the cytotoxic B3Z cells. Since the new B3Z cells had been demonstrated to be activated by SIINFEKL presented in the MHC I, an increase in the presentation of EVI through glycosylation should be observable. Given that the SIINFEKL peptide was the only antigen to be detected by the B3Z cells in this study, it was also glycosylated through conjugation to oxidized mannan to determine if its presentation would be improved.

Methods

Conjugating Mannan to antigens

Oxidized mannan (Sigma) was conjugated to EVI or SIINFEKL according to Apostolopoulos *et al.* (1995) as described previously. Mannan was oxidized by sodium periodate and eluted through a HiTrap Q Sepharose HP column (GE Healthcare) equilibrated with sodium bicarbonate buffer (pH 9.0) to elute only the oxidized

mannan. The oxidized mannan was then incubated with antigen overnight at room temperature. The resulting glycosylated protein was assayed for the degree of glycosylation.

Assays for glycosylation of antigens

The glycosylated proteins were assayed for glycosylation by electrophoresis and using a glycoprotein detection kit (Pierce). The proteins were first assayed using electrophoresis as described previously to determine if the glycosylated antigen migrated at a different rate compared to the non-glycosylated antigen due to the added molecular weight of the conjugated mannose.

A glycoprotein carbohydrate estimation kit (Pierce) was used to determine the amount of glycosylation of the proteins. To test for carbohydrate content, 50 μ L of standards and samples were added to a 96 well plate. Controls were glycoprotein detection reagent, oxidized mannan and PBS. Twenty-five microliters of 10 mM sodium meta-periodate (prepared just prior to use) was added to each well and mixed for 30 seconds on a plate shaker. After incubating for 10 min at room temp, 150 μ L of glycoprotein detection reagent (prepared just prior to use) was added to each well and mixed for 30 seconds. The plate was incubated at room temp for one hour before measuring absorbance at 560 nm using the Modulus Microplate Reader.

The absorbance of the standards was then compared to the absorbance of the samples and controls. A standard curve was produced by subtracting the absorbance for the blank (glycoprotein detection reagent control) from the absorbance of the protein

standards. The absorbance of the protein standards with the blank subtracted was then plotted with carbohydrate content (%) based on the values provided by the manufacturer. The plot was fit for a linear regression and the resulting equation was used to calculate the carbohydrate content of the samples. If the absorbance of the sample was above 5.00, the carbohydrate content could not be calculated since the exact absorbance was too high to be read on the microplate reader.

In addition, the amount of protein in both the glycosylated and non-glycosylated samples was quantified using the Qubit Fluorometer as described previously to determine the concentration of protein in the solutions.

B3Z assay using RPMI

The glycosylated EVI and SIINFEKL were then assayed for cross-presentation by activation of B3Z cells using the RPMI method as described previously. The glycosylated and non-glycosylated antigens were mixed with splenocytes and B3Z cells and incubated for 24 hrs. The controls were oxidized mannan, diluted oxidized mannan (at the same dilution as the glycosylated SIINFEKL, 1:60) and PBS. The β -gal production was then determined using the Luminescent β -gal detection kit II (Clontech) and the Modulus Microplate Reader.

Results

The antigens EVI and SIINFEKL were conjugated to oxidized mannan as described by Apostolopoulos *et al.* (1995). To determine if the proteins were glycosylated, a gel

electrophoresis was conducted on the glycosylated proteins and the non-glycosylated proteins. When the electrophoresis was performed, the bands for the glycosylated EVI were not visible on the gel (Figure 19A). The lanes containing the glycosylated EVI had a dark stained area where the protein was loaded into the well. It was hypothesized that the glycosylated EVI was too large to enter the gel. The bands in the lanes with glycosylated SIINFEKL did appear to shift slightly compared to the non-glycosylated SIINFEKL. The bands are very faint, but when looking at the leading edge of the glycosylated band compared to the leading edge of the band for the non-glycosylated SIINFEKL, it did not migrate as far down the gel, (Figure 19 B).

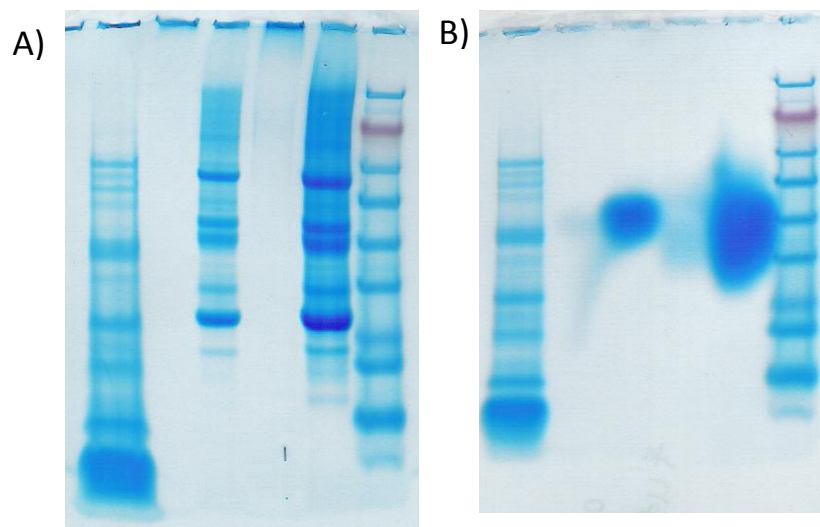


Figure 19: Electrophoresis of Glycosylated Antigens. The glycosylated proteins were electrophoresed on a Tris-Glycine gel along with the non-glycosylated version of the protein to determine if glycosylation had occurred. Panel A depicts the gel with EVI. From left to right, lane 1 is the molecular weight standard Mark 12 (Invitrogen). Lanes 2 and 4 are the glycosylated EVI at 5 and 15 μ L respectively. Lanes 3 and 5 are the non-glycosylated EVI at 5 and 15 μ L respectively, with the EVI protein appearing as the darkest band closest to the wells. Lane 6 is the molecular weight standard See Blue (Invitrogen). Panel B shows the gel with SIINFEKL. The lanes were set up as described for the EVI but with glycosylated and non-glycosylated SIINFEKL.

EVI and SIINFEKL were both tested for glycosylation using the Glycoprotein Carbohydrate Estimation Kit (Pierce). The non-glycosylated EVI and SIINFEKL were also tested for carbohydrate content. A standard curve was prepared from a set of standard glycoproteins (Figure 21). The linear regression for the standard curve was found to be:

$$\text{Absorbance} = 0.2512 (\% \text{ Carbohydrate}) + 0.5857$$

Using this formula, the carbohydrate content of the EVI and SIINFEKL were calculated.

The EVI (non-glycosylated) was found to be 1.3% glycosylated, while the EVI conjugated to mannan could not be calculated because the absorbance was over 5.00 and the absorbance could not be determined by the microplate reader. The SIINFEKL was estimated to be 0% glycosylated, since its absorbance was only off by 0.01 from the blank, while the SIINFEKL conjugated to mannan could not be calculated because the absorbance was over 5.00 (Table 1).

Table 1: Estimation of carbohydrate content of sample proteins. The carbohydrate content of non-glycosylated EVI and SIINFEKL and glycosylated EVI and SIINFEKL was found using a glycoprotein estimation kit. The absorbance of the samples was found at 560nm. The blank of glycoprotein detection reagent was subtracted from each absorbance and this value was used to compare the sample to the absorbance of standard glycoproteins with known carbohydrate content which also had the blank subtracted from them. Samples with an absorbance over 5.00 could not be estimated since the exact absorbance could not be detected by the microplate reader.

Samples	Protein (mg/mL)	Absorbance (560 nm)	Absorbance – Blank	Estimated Carbohydrate Content (%)
Blank	0	3.92	0	0
EVI	2.5	4.82	0.91	1.3
EVI	0.25	3.61	-0.30	-
Glycosylated EVI	0.45	5.00	1.08	-
Glycosylated EVI	0.045	5.00	1.08	-
Glycosylated SIINFEKL	0.45	5.00	1.08	-
Glycosylated SIINFEKL	0.045	5.00	1.08	-
SIINFEKL	2.5	3.93	0.01	0
Mannan	0	5.00	1.08	-
Mannan	diluted 1:10	5.00	1.08	-

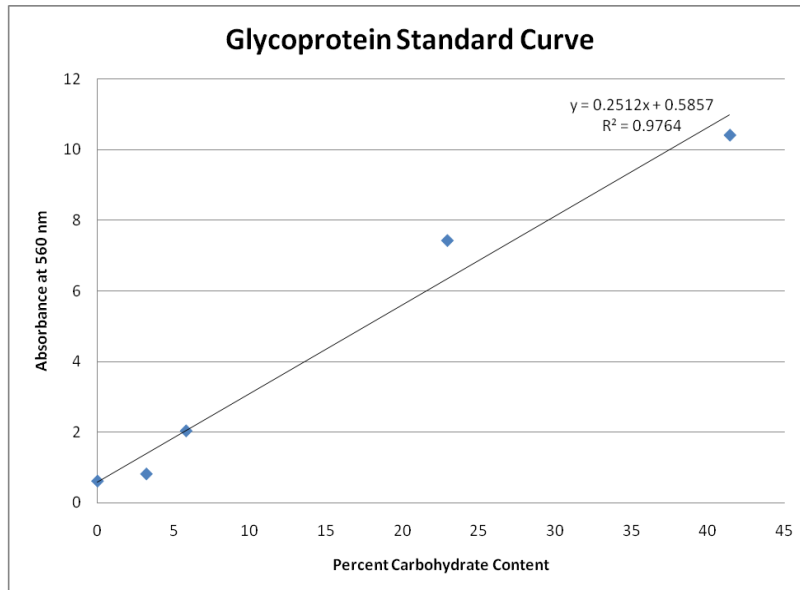


Figure 20: Glycoprotein Standard Curve. A glycoprotein standard curve was found using the glycoprotein standards Lysozyme, Bovine Serum Albumin, Ovalbumin, Apo-Transferrin, Fetuin, and α_1 -Acid Glycoprotein and the glycoprotein detection reagent. Samples were mixed with sodium meta-periodate and detection reagent. The absorbance at 560 nm was then measured.

It was uncertain whether the glycoprotein detection reagent was detecting glycosylated protein or just the mannan still in solution. Since the protein bands for the glycosylated EVI did not appear on the gel electrophoresis, the protein content of the glycosylated EVI and SIINFEKL was measured using the Qubit fluorometer (Invitrogen) to determine the protein concentration. The glycosylated EVI was found to be 199.4 $\mu\text{g/mL}$, while the non-glycosylated EVI at the same dilution was 390 $\mu\text{g/mL}$. The glycosylated SIINFEKL was found to be 196.2 $\mu\text{g/mL}$, while the non-glycosylated SIINFEKL at the same dilution was found to be 250 $\mu\text{g/mL}$.

Finally, the glycosylated and non-glycosylated proteins were assayed for presentation by splenocytes using the RPMI method. Both the glycosylated and non-glycosylated SIINFEKL resulted in significant β -gal production compared to the controls of diluted mannan and PBS respectively. The glycosylated SIINFEKL approached a significant difference at the highest concentration compared to non-glycosylated SIINFEKL ($p = 0.078$). The glycosylated EVI also showed significantly greater β -gal production when compared to the oxidized mannan control and the non-glycosylated EVI at each concentration. The non-glycosylated EVI did not have significant β -gal production above the PBS control (Figure 22). The glycosylated EVI also had significantly greater β -gal production than the glycosylated SIINFEKL and non-glycosylated SIINFEKL at concentrations of 3.93, 1.96, 0.98 and 0.49 μM .

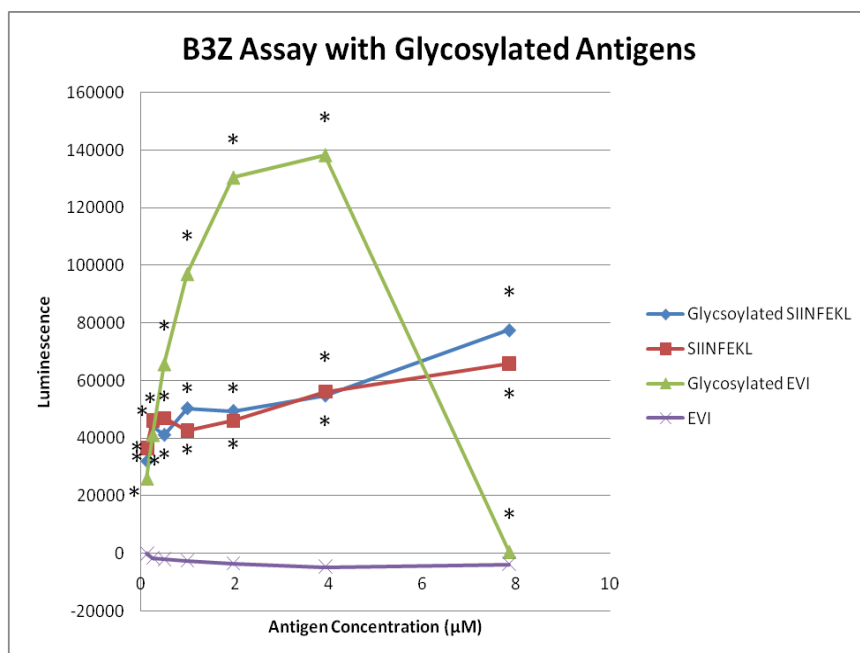


Figure 21: B3Z Assay with Glycosylated Antigens. A B3Z assay was conducted using the RPMI method with glycosylated SIINFEKL and EVI compared to non-glycosylated SIINFEKL and EVI. Significance is indicated by stars (*) ($p < 0.05$). All glycosylated EVI readings are also significantly greater than the non-glycosylated EVI.

Discussion

The antigens EVI and SIINFEKL were conjugated to oxidized mannan, as described by Apostolopoulos *et al.* (1995), in attempt to increase their cross-presentation. To test if the antigens were glycosylated, the proteins were separated by electrophoresis. Both glycosylated and non-glycosylated versions of EVI and SIINFEKL were assayed. If the proteins were glycosylated, the protein band should not migrate as far on the gel due to the added size of the conjugated mannan.

A band in the lane loaded with glycosylated EVI was not evident on the gel. There was however a dark area in the well of the lane loaded with 15 μL of glycosylated EVI, suggesting that the EVI protein may have been glycosylated and was too large to enter the gel. The glycosylated SIINFEKL bands were very faint compared to the non-

glycosylated SIINFEKL, but the leading edge of the glycosylated band appeared to have migrated slightly less compared to the leading edge of the non-glycosylated band. It was difficult to tell if this was a significant difference, as the non-glycosylated SIINFEKL bands were very wide, presumably due to the large concentration of SIINFEKL in the sample.

Since the electrophoresis did not irrefutably confirm glycosylation, a glycoprotein estimation kit was used to determine the carbohydrate content of the proteins. Both glycosylated and non-glycosylated EVI and SIINFEKL were assayed for carbohydrate content, along with the control of oxidized mannan. According to the standard curve formed from glycoprotein standards, EVI that was not conjugated to mannan had slight glycosylation. The SIINFEKL was estimated to not be glycosylated, since its absorbance was only 0.01 more than the blank.

The oxidized mannan control also gave an absorbance over 5.00, indicating the estimation kit was not identifying glycoproteins as a whole, but instead corresponded to the carbohydrates in solution. For this reason, the carbohydrate content attached to the EVI and SIINFEKL could not be estimated. The mannan in solution could be causing the large absorbance rather than mannan conjugated to the proteins.

To assay if the proteins were still in solution or to determine if they had been degraded in the conjugation process, since the bands had not appeared or were very faint on the electrophoresis gels, the concentration of protein for each antigen solution was found using the Qubit flurometer. The glycosylated EVI and SIINFEKL solutions had concentrations of 199.4 µg/mL and 196.2 µg/mL respectively. The non-glycosylated EVI

and SIINFEKL were assayed to be 390 µg/mL and 250 µg/mL respectively. Each solution should have had a concentration of 450 µg/mL as this was what the non-glycosylated proteins were diluted to prior to the assay. The glycosylated proteins were also diluted to this concentration when mixed with the oxidized mannan to undergo glycosylation. This indicated that there was a slight loss of protein through the glycosylation procedure. This could explain the faintness of the SIINFEKL bands on the gel. This also reinforces the hypothesis that EVI is glycosylated, since there is still protein present in solution, but the band did not appear on the gel. This was most likely due to the glycosylated EVI being too large to enter the gel.

With moderate certainty that the EVI and SIINFEKL were glycosylated by the mannan conjugation procedure, they were assayed for their ability to be cross-presented and activate the B3Z cell line using the RPMI method. The glycosylated SIINFEKL showed similar β-gal production compared to the non-glycosylated SIINFEKL, until the highest concentration of antigen. At the highest concentration, the glycosylated SIINFEKL approached a significantly greater β-gal production compared to the non-glycosylated SIINFEKL ($p = 0.078$).

The EVI showed a significant difference in β-gal production at all concentrations when glycosylated compared to the non-glycosylated. It was also the first time, during this study, that EVI showed a significant increase in β-gal production compared to the control. The glycosylation appeared to increase the cross-presentation of the EVI, thereby increasing the number of B3Z cells that were activated and the amount of β-gal

produced. This supported the hypothesis that glycosylation of the HER2 protein EVI would increase cross-presentation.

Cross-presentation of EVI was achieved through glycosylation, and the amount of β -gal produced by B3Z cells in response was even greater than both the glycosylated and non-glycosylated SIINFEKL peptide at four concentrations. This suggests that through glycosylation, an antigen can be processed by the cell and presented more efficiently than the peptide alone. When considering a glycosylated antigen as a vaccine vector, it would have not only the advantage of increasing presentation of a specific antigenic peptide, but also allow for presentation of other peptides within the whole protein. It would be hypothesized that since glycosylation was able to increase the presentation of SIINFEKL, it would also increase the presentation of other peptides formed from the processing of the HER2 protein.

CHAPTER 7: PROTEIN TRANSFECTION AGENTS

Introduction

The goal of this project was to increase cross-presentation of the tumor antigen HER2. To accomplish this, the protein needs to be delivered from the extracellular environment into endosomes or the cytosol so that it enters into a MHC I presentation pathway. Since antigens presented in the MHC I come from within the cytosol, HER2 antigen delivered directly into the cytosol by a transfection agent should increase the cross-presentation.

For this reason, several different protein transfection agents were assayed for their ability to increase the cross-presentation of the HER2 protein EVI. The protein transfection agents are designed to bind to proteins and chaperone them across the cell membrane, into the cell. Once inside the cell, the transfection agents release the intact, still functional protein. If the protein transfection agents deliver the protein across the membrane, it would be more likely for the protein to be processed as an internal antigen and therefore be presented in the MHC I.

The protein transfection agents assayed for their ability to increase cross-presentation were TransPass-P (New England Biolabs), jetPRIME PEI (Polyplus-transfection) and PULSin (Polyplus-transfection). All of these agents have been demonstrated to successfully transfect living cells with minimal toxicity, according to the manufacturers. The TransPass-P protein transfection agent forms a non-covalent bond with the protein to deliver the protein into mammalian cells by endocytosis. The

non-covalent association prevents protein degradation within the endosome when endocytosed (New England Biolabs).

The jetPRIME (PEI) is a transfection agent intended for DNA transfection, but was assayed for its ability to bind and transfect protein. Polyethyleneimine (PEI) is a synthetic branched polymer that has a high cationic charge density. The cationic PEI forms noncovalent interactions with DNA, and promotes cellular uptake by endocytosis (Grezelski *et al.*, 2006). PEI has been also shown to bind and deliver proteins into living cells *in vitro* and *in vivo*. When the PEI binds to the protein, the protein becomes cationized and adheres to the cell surface through an ionic charge interaction. The PEI/protein complex is internalized by the cells without binding to a receptor or transporter (Futami *et al.*, 2005). Since PEI has been used as a protein transfection agent, the jetPRIME was assayed for its transfection ability with the EVI protein.

The third transfection agent used was PULSin (Polyplus-transfection). PULSin is an amphiphile cationic molecule that also forms non-covalent bonds with the protein. It binds to anionic cell-adhesion receptors on the cell surface, which are then internalized. Once in the cell, the complexes of PULSin/protein are released into the cytoplasm and disassemble (Polyplus-transfection). If PULSin is able to deliver the EVI protein directly into the cytoplasm, it should get processed as an internal antigen and be presented in an MHC I, thereby achieving cross-presentation.

Methods

TransPass-P protein transfection agent

Splenocytes were isolated as described previously into serum free AIM-V media. The antigens were mixed with the transfection agent TransPass-P (New England BioLabs) as described by the manufacturer. Each antigen was diluted to 10 μ M and mixed with the TransPass-P for 20 min at room temp. The resulting transfection mix was added to the splenocytes and incubated at 37 °C and 5% CO₂ for 4 hrs. B3Z cells were added to the plate in IMDM media as described for the super-activating splenocytes procedure. The plate was incubated for an additional 24 hrs at 37 °C and 5% CO₂ before determining the β -gal production using the Luminescent β -gal Detection Kit II (Clontech) and the Modulus Microplate Reader.

jetPRIME transfection agent:

Splenocytes were isolated into complete RPMI media as described previously. Each antigen was diluted to a concentration of 1 μ g/mL in PBS. To make the transfection mixes, 2 μ L of diluted antigen, 4 μ L jetPRIME (Poly Plus Transfection) and 100 μ L jetPRIME buffer were mixed together and incubated at room temperature for 10 min. The transfection mix was added to the splenocytes and incubated overnight at 37 °C and 5% CO₂. B3Z cells in complete RPMI were added to the plate and incubated an additional 24 hrs at 37 °C and 5% CO₂ before determining the β -gal production using the Luminescent β -gal Detection Kit II (Clontech) and the Modulus Microplate Reader.

PULSin protein transfection agent

Splenocytes were isolated into complete RPMI as described previously. Before adding the cells to the plate, they were washed with complete serum free RPMI to remove serum. Each antigen was diluted to a concentration of 1 µg/mL in PBS. To make the transfection mixes, 2 µL of antigen diluted to 1 µg/mL, 4 µL PULSin transfection agent (Poly Plus Transfection) and 100 µL 20mM HEPES buffer were mixed together and incubated at room temperature for 15 min. Transfection mix was added to the splenocytes and incubated overnight at 37 °C and 5% CO₂. B3Z cells in complete RPMI media were added to the plate and incubated for 24 hours at 37 °C and 5% CO₂ before determining the β-gal production using the Luminescent β-gal Detection Kit II (Clontech) and the Modulus Microplate Reader.

Cell Viability Assays

Splenocytes and B3Z cells were assayed for viability in the presence of each protein transfection agent and the buffer used to make the transfection mix, if applicable. Wells with media alone and cells alone were used as controls. The cells were added to the plate in the same media and incubated for the same period of time as described for each transfection agent. The cell viability was then assayed using the Cell Titer Glo Luminescent Assay (Promega) and the Modulus Microplate Reader. The reagent measures cell viability by ATP production.

Results

The first protein transfection agent applied in attempt to deliver the antigens into the splenocytes for presentation as an internal antigen was TransPass-P (New England Biolabs). When SIINFEKL was presented with TransPass-P or alone, the β -gal production was greater than the TransPass-P or PBS controls. In the presence of TransPass-P however the SIINFEKL had a significantly small β -gal production when compared to SIINFEKL without TransPass-P (Figure 22A). The TransPass-P did not have a significant effect on OVA presentation, as neither the OVA with TransPass-P or OVA alone were significantly above the controls. It appeared that the β -gal produced by OVA presentation was lower when presented with TransPass-P, though not statistically different (Figure 22B).

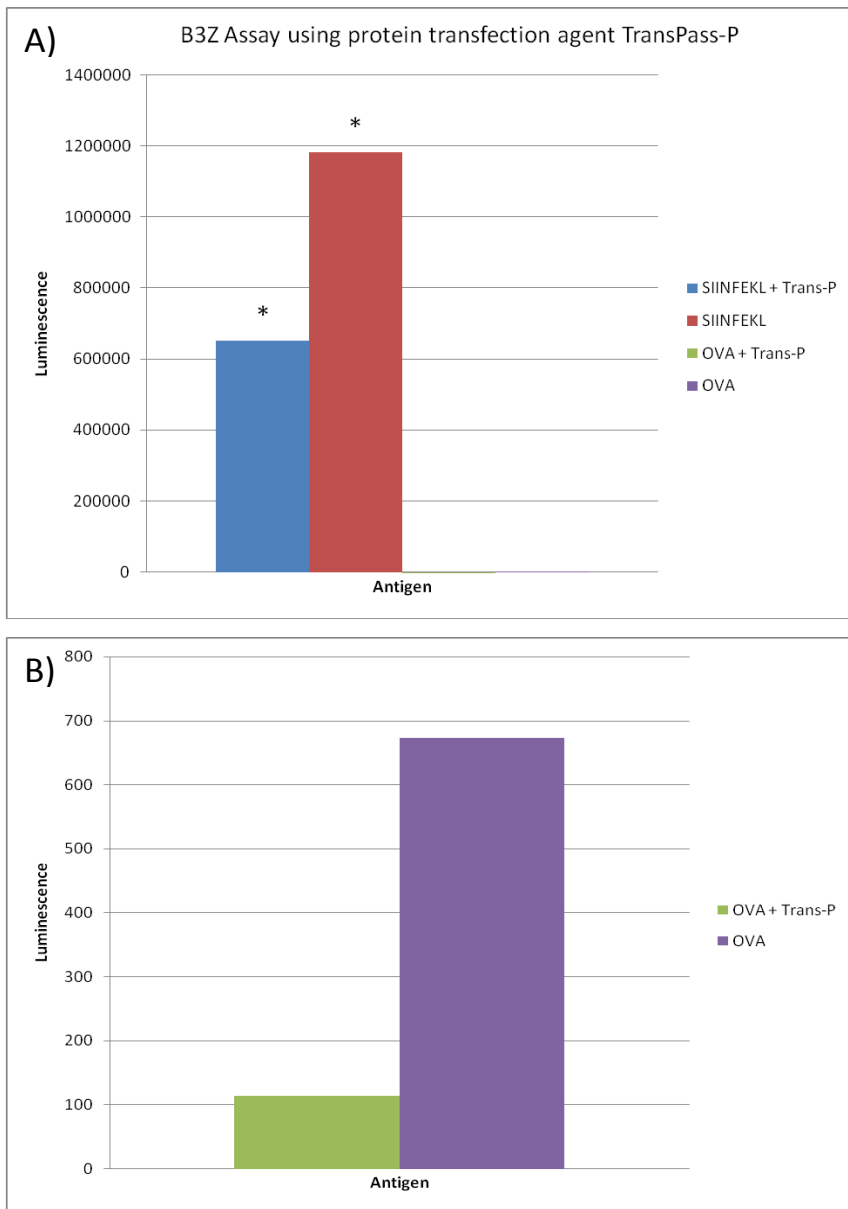


Figure 22: B3Z Assay using the Protein Transfection Agent TransPass-P.

Splenocytes were incubated with TransPass-P protein transfection agent and antigen for 4 hours before adding B3Z cells. The β -gal production was then measured as a luminescent reading. Readings significantly ($p \leq 0.05$) greater than the PBS control or the TransPass-P without antigen control are indicated by the stars (*). The SIINFEKL and SIINFEKL + TransPass-P differed significantly. Panel A shows all antigens while panel B shows only OVA.

A cell viability assay to determine the amount of ATP present was also conducted to determine if the TranPass-P had an effect on cell proliferation. There was no significant difference in the cell viability of splenocytes and B3Z cells when mixed with TranPass-P compared to cells that were not (Figure 23).

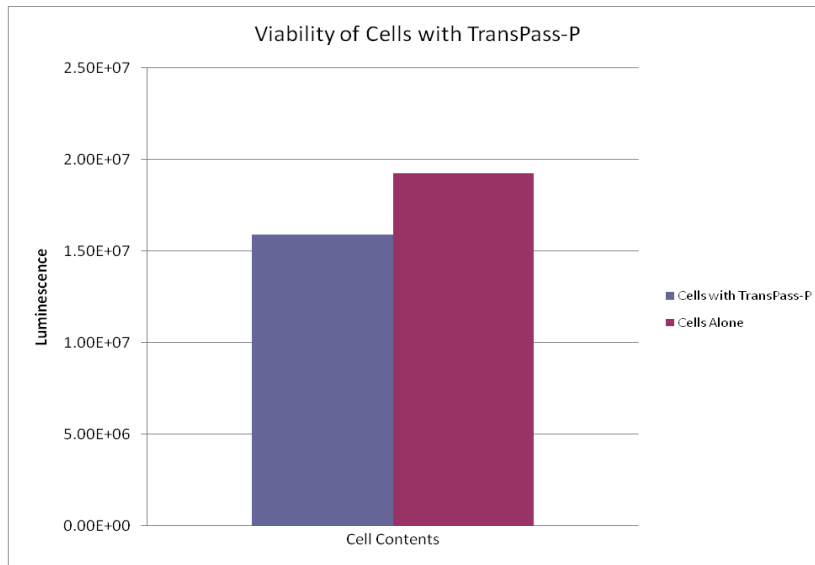


Figure 23: Viability of cells with TransPass-P.

Cell Viability of splenocytes and B3Z cells in the presence of TransPass-P was measured by ATP production through a cell titer glo luminescent assay. There was no significant difference ($p \leq 0.05$) between cells in the presence or absence of TransPass-P.

The jetPRIME (PEI) transfection agent (Polyplus-transfection) was next assayed for its ability to transfect the antigens SIINFEKL, OVA and EVI. The SIINFEKL peptide gave significant β -gal production above the jetPRIME buffer control and the jetPRIME with jetPRIME buffer control when combined with jetPRIME or SIINFEKL alone. In combination with the jetPRIME, the β -gal production was significantly lower than the SIINFEKL presented in jetPRIME buffer alone (Figure 24A). When presented with the jetPRIME buffer, OVA and EVI both were able to stimulate β -gal production significantly greater than the control of jetPRIME buffer. In combination with jetPRIME transfection agent, OVA and EVI did not stimulate significant β -gal production greater than the jetPRIME control. For EVI, not only was the β -gal production not significant, but the β -gal production was less than the control (Figure 24B).

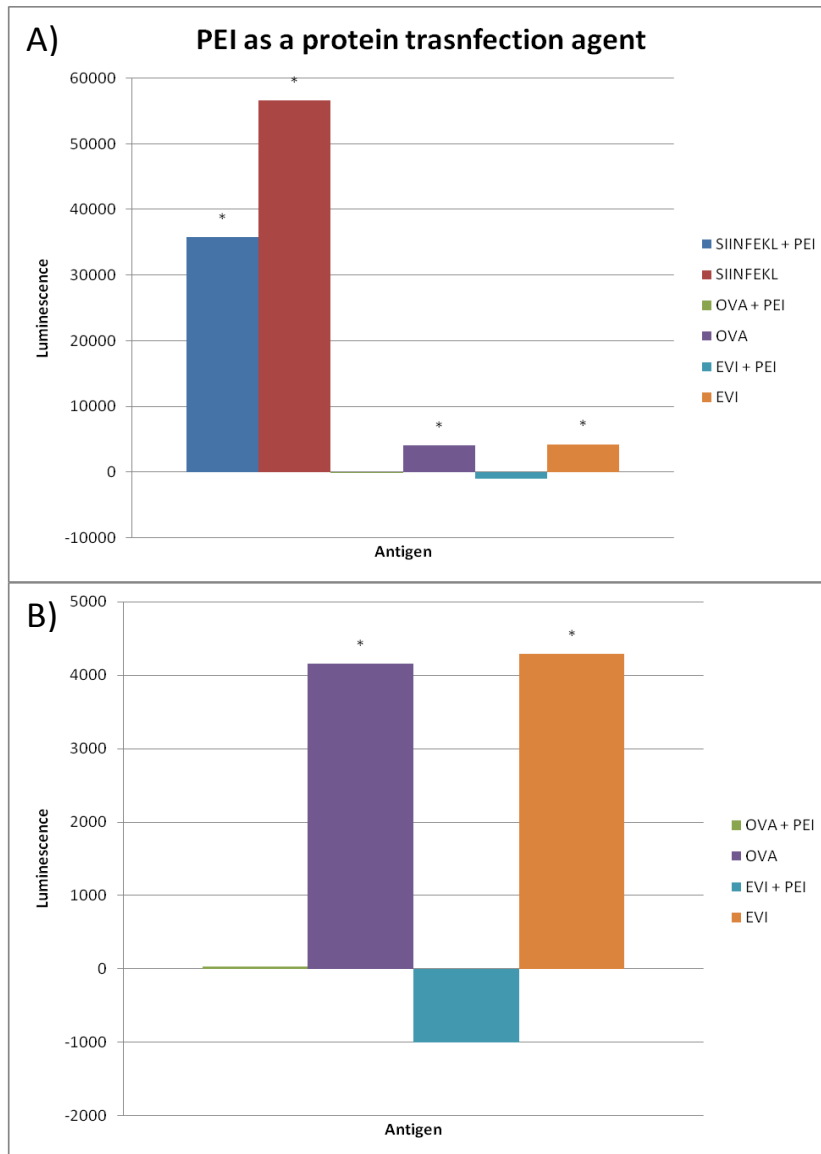
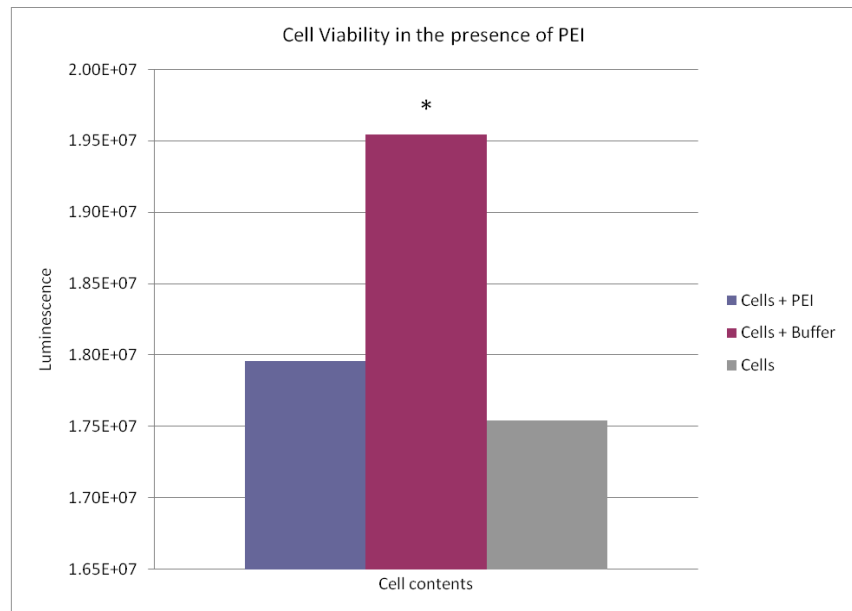


Figure 24: PEI as a protein transfection agent. The PEI transfection agent jetPRIME was incubated with splenocytes and antigen overnight before adding B3Z cells. The β -gal production was then measured as a luminescent reading. Readings significantly ($p \leq 0.05$) above the jetPRIME buffer control or the jetPRIME control without antigen are indicated by the stars (*). The luminescence from β -gal production in cells presented with each antigen was also significantly higher than antigen with jetPRIME. Panel A shows all antigens. Panel B shows only OVA and EVI.

A cell viability assay was also conducted on the cells with jetPRIME to determine if it had any cell cytotoxicity that would account for the decrease in β -gal production by B3Z cells in the presence of jetPRIME compared to control. While the cells with PEI (jetPRIME) did not vary significantly from the cells alone, the cells with jetPRIME buffer had significantly more luminescence than either the cells alone or the cells with jetPRIME (Figure 25).

Figure 25: Cell viability in the presence of PEI. Cell viability of splenocytes and B3Z cells in the presence of jetPRIME (PEI) was measured by ATP production using a luminescent assay. There was no significant difference ($p \leq 0.05$) between cells in the presences of PEI and control cells. The cells in the presence of jetPRIME buffer were



significantly different from the control cells and cells with jetPRIME as indicated by the star (*).

The protein transfection agent PULSin (Polyplus-transfection) was assayed for its ability to transport the proteins into the cytoplasm of the antigen presenting cells and therefore increase cross-presentation of the antigens. When SIINFEKL was presented with PULSin or alone it gave β -gal production above the PULSin control or PULSin buffer without antigen. In the presence of PULSin, the β -gal production was significantly lower than SIINFEKL presented without PULSin. The OVA with PULSin stimulated production of significant amounts of β -gal compared to PULSin alone, but the OVA with only the PULSin buffer was not significantly higher than the control of PULSin buffer. The β -gal production in response to OVA in the presence of PULSin was also significantly higher than OVA alone (Figure 26).

The EVI with PULSin or EVI alone (with PULSin buffer) did not stimulate a significant β -gal production above the PULSin control or PULSin buffer control respectively. The EVI alone resulted in significantly higher B-gal production than EVI with PULSin, which resulted in β -gal production lower than its control (Figure 26).

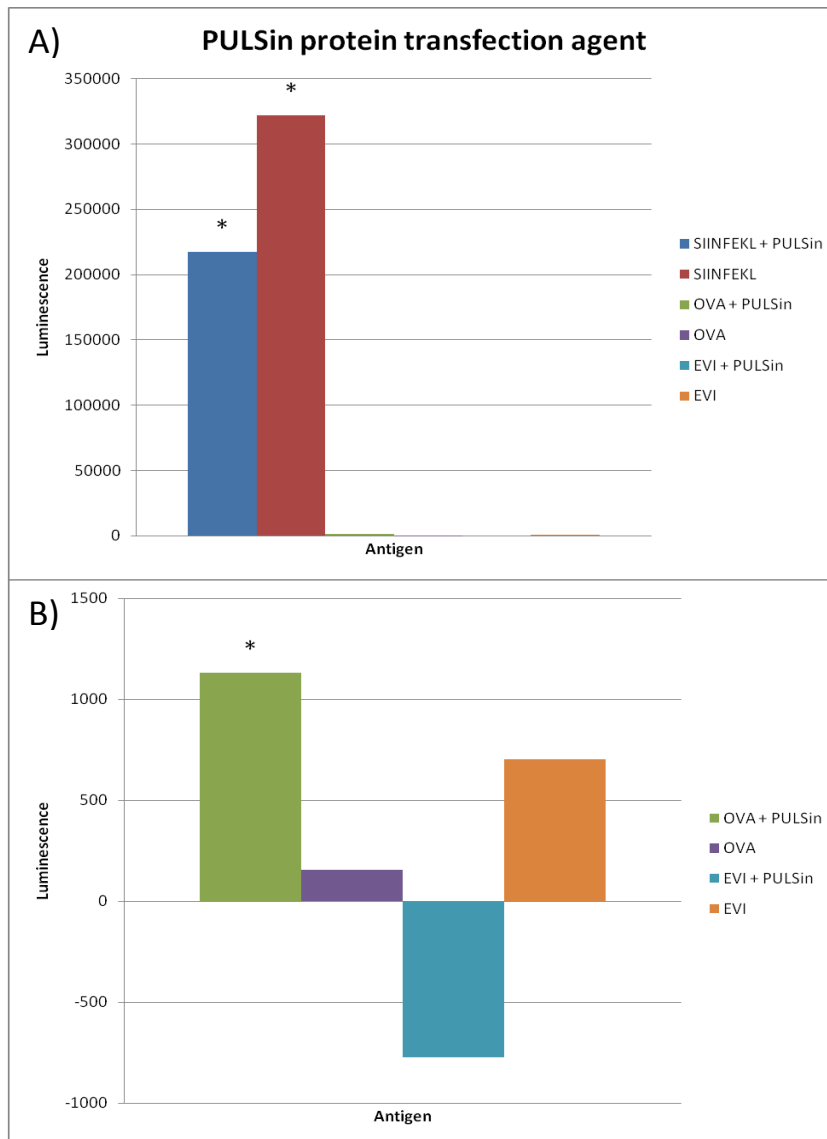
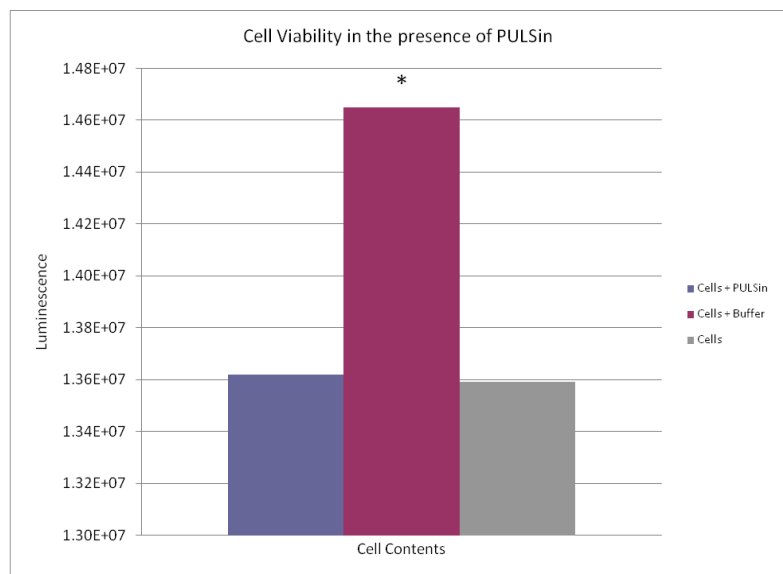


Figure 26: PULSin as a protein transfection agent. The protein transfection agent PULSin was incubated with splenocytes and antigen overnight before adding B3Z cells. The β -gal production was then measured as a luminescent reading. Significant readings compared to the controls (buffer or PULSin without antigen) are indicated by the stars (*) ($p \leq 0.05$). The luminescence from β -gal production in cells presented with SIINFEKL or EVI was significantly higher than SIINFEKL or EVI with jetPRIME. The β -gal produced by cells with OVA and PULSin was significantly higher than OVA alone. Panel A shows all antigens while panel B shows only OVA and EVI.

When the cell viability assay was conducted on splenocytes and B3Z cells in the presence of the PULSin protein transfection agent or the buffer, the cells incubated with PULSin were not significantly different from the cells alone. Luminescence resulting from ATP production in cells with PULSin buffer however was significantly greater than both the cells with PULSin and the cells alone (Figure 27).

Figure 27: Cell viability in the presence of PULSin. Cell Viability of splenocytes and B3Z cells in the presence of PULSin was measured by ATP production through a luminescent assay. There was no significant difference between cells in the presence of PULSin and control cells. The cells in the presence of PULSin buffer were significantly different from the control cells and cells with PULSin as indicated by the star (*) ($p \leq 0.05$).



Discussion

Several different transfection agents were assayed for their ability to increase the cross-presentation of the antigens SIINFEKL, EVI and OVA. The transfection agents form noncovalent interactions with the proteins and help to transport them across the cell membrane. Once the protein is inside the cell, it was hypothesized that it would be processed as an internal antigen and presented in the MHC I. If this was achieved by the protein transfection agents, presentation of the antigens in the presence of the

transfection agent should increase the amount of β -gal produced by the B3Z cells as a result of activation of the B3Z cells from presentation of the peptide SIINFEKL in the MHC I of the splenocytes.

The protein transfection agent TransPass-P caused a decrease in β -gal production when the antigens were presented with this agent. The SIINFEKL was able to be presented and caused a significantly higher luminescence as a result of β -gal production compared to the controls of TransPass-P or PBS alone. The TransPass-P significantly decreased the β -gal production compared to SIINFEKL alone. This appeared to be the case for OVA as well, even though the difference was not statistically significant. The TransPass-P did not increase cross-presentation, but instead seemed to inhibit cross-presentation. This difference was not due to toxicity of the cells, as the cells with TransPass-P gave a similar luminescence in the cell viability assay compared to the cells alone.

The transfection agent jetPRIME, a variation of PEI, had similar results. The jetPRIME significantly reduced the amount of β -gal produced as a result of SIINFEKL presentation when compared to SIINFEKL alone, even though both values were again significantly above the controls. This decrease cannot be attributed to toxicity, as the cells alone and with jetPRIME were not significantly different in the cell viability assay.

The EVI and OVA stimulated β -gal production significantly above the controls when presented alone. Presentation of EVI and OVA with PEI significantly reduces β -gal production. Interestingly, the jetPRIME buffer combined with the antigens (but not jetPRIME) stimulated β -gal production in the B3Z cells. Additionally, increased cell

viability occurred when the cells were incubated with jetPRIME buffer compared to jetPRIME or no treatment. However, this difference is accounted for when the cells with buffer alone control is subtracted from the luminescent reading of the cells with antigen and buffer.

As with the other transfection agents, PULSin caused a significant decrease in β -gal production when mixed with SIINFEKL compared to SIINFEKL alone. The SIINFEKL presentation was still significantly greater than the controls in both cases. The EVI showed a similar pattern in that it caused more β -gal production alone than in conjunction with the PULSin. As with the jetPRIME, this may be attributable to the fact that the cells with buffer alone had increased cell viability compared to cells alone or with PULSin. The decrease is not a result of toxicity of the PULSin, as the cells with PULSin were not significantly different from the cells alone during the cell viability assay.

The OVA in combination with PULSin was the only antigen/protein transfection agent combination that displayed significant β -gal production when compared to the antigen alone. The OVA and PULSin also stimulated a significantly greater luminescence reading, from β -gal production, when compared to the PULSin alone control. This suggests that the PULSin was able to bind and transport OVA into the cell, increasing the amount of cross-presentation.

In most cases the transfection agents significantly decreased presentation of the antigens, as observed by a decrease in β -gal production when in combination with the transfection agent. This decrease could be a result of the transfection agent binding, but not releasing the antigen once in the cell, or it could also be caused by the antigen

getting endocytosed to get into the cell, but then entering the MHC II pathway, since most extracellular antigens that are endocytosed get presented in the MHC II. Instead of increasing cross-presentation, the transfection agents may have been simply increasing MHC II presentation, which is not detected with this assay.

The OVA increase in cross-presentation however demonstrates that it is possible to use protein transfection agents to increase cross-presentation. As shown by the decrease in EVI presentation when using the same transfection agent, it implies that the right transfection agent for each antigen must be found. Since proteins are very different from one another, compared to DNA or RNA which have the consistent backbone structure, it would be important to find a transfection agent that is able to bind the protein based on its unique amino acid composition. Not only must it be able to bind the protein, but it also must be able to release it once it gets into the cell. A more serious study of the characteristics of HER2 or other antigens and the properties of various transfection agents may lead to the discovery of agents that are able to increase cross-presentation as was observed with OVA.

SYNOPSIS AND CONCLUSIONS

HER2 overexpression in breast cancer leads to a very aggressive form of the disease. It has been correlated with decreased survival and faster progression when compared to breast cancers not overexpressing HER2. For this reason, HER2 has proven to be an excellent target for treatment, as the cancer cells not only depend on HER2 for survival, but HER2 has also been shown to be the initial cause of transforming normal cells into cancer (Moasser, 2007a). Its presence has also been correlated to a population of cancer stem cells, which impel tumorigenesis (Korkaya *et al.*, 2008). By targeting HER2 in treatments, the driving force of the cancer can be eliminated, effectively preventing the growth of cells overexpressing the receptor.

The first treatment to take advantage of this concept was the monoclonal antibody trastuzumab. Although the development of trastuzumab has led to a longer onset of disease progression and lower death rate at one year (Slamon *et al.*, 2001), a relatively large fraction of patients still relapse and one-third of the patients with advanced forms of the disease fail to respond to the treatment (Korkaya *et al.*, 2008). This along with the severe side effect of cardiac dysfunction in 4.7% of patients treated with trastuzumab (Eisenhauer, 2001) encourages the development of a novel treatment for targeting HER2 in breast cancer.

A treatment option that has recently been explored is the formation of a vaccine against HER2. Evidence has shown that HER2 could be a possible candidate for a

vaccine, despite it being a self-protein. Patients with HER2 overexpressing tumors have a pre-existing, though low magnitude, immune response to HER2 (Bernhard *et al.*, 2002). A vaccine to boost this pre-existing immunity or elicit a novel immunity against HER2 could be the next step at developing a more effective treatment for HER2 positive breast cancer.

The optimal immune response to a tumor vaccine is the activation of cytotoxic T cells. These cells are specific to antigens on the surface of cells and target them for destruction. Cytotoxic T cells have demonstrated to be required for tumor rejection by the immune system (Rohrback *et al.*, 2005). In order for a vaccine to activate cytotoxic T cells, the antigens composing the vaccine need to be presented in a MHC I. This requires the antigen to be cross-presented from the extracellular environment to the internal antigen processing pathway.

The goal of this project was to optimize the cross-presentation of the tumor protein HER2. Glycosylation has shown to increase antigen presentation in general (Tan *et al.*, 1997), as well as to specifically increase the amount of cross-presentation to elicit a cytotoxic T cell response (Apostolopoulos *et al.*, 1995). To determine if this was true for HER2, a variation of the HER2 protein, EVI, was conjugated to oxidized mannan. The glycosylated EVI demonstrated an increased cytotoxic T cell response when glycosylated compared to non-glycosylated as measured by the activation of the cytotoxic T cell line B3Z through production of β -gal. Glycosylated EVI caused significantly greater β -gal production compared to non-glycosylated EVI. This data supports previous studies demonstrating that glycosylation of a protein increased cross-

presentation. Evidence from this study, as well as from others, indicates that glycosylated HER2 antigens should be considered as a viable construct for an effective tumor vaccine.

The glycosylated EVI was able to cause significant β -gal production, even when compared to the β -gal produced by the glycosylated and non-glycosylated SIINFEKL peptides at four concentrations. This demonstrated that through glycosylation, an antigen can be presented more efficiently than the peptide alone, increasing the number of cytotoxic T cells activated in response to that antigen. A glycosylated antigen as a vaccine vector, would have not only the advantage of increasing presentation of a specific antigenic peptide, as demonstrated with SIINFEKL presentation, but also allow for other peptides within the whole protein to be processed and presented. It could be hypothesized that since glycosylation was able to increase the presentation of SIINFEKL, it would also increase the presentation of other peptides formed from the processing of the HER2 protein.

The B3Z cells are specifically activated by the SIINFEKL peptide presented in the MHC I. In order for EVI and OVA to cause β -gal production, they needed to be cleaved into fragments and the SIINFEKL peptide presented in the MHC I to elicit B3Z cell activation. Since the SIINFEKL peptide produced more β -gal at the same concentrations of antigen as the proteins, it illustrated that the peptide was more efficiently in to causing activation than the whole proteins, except when the EVI was glycosylated. This demonstrates that peptides capable of direct presentation in the MHC I, without being

cleaved by proteosomes or digestive enzymes within the lysosome, can be efficiently presented and elicit a strong response by cytotoxic T cells.

Data from this study indicates that while the peptide is ready to be presented in an MHC I, it still needs to be internalized by the cell and undergo cross presentation in order to cause a significant T cell response, as was observed when the splenocytes were fixed with glutaraldehyde. The cells that were fixed and not able to process the antigen internally gave significantly less β -gal production compared to cells that were not fixed and could process the antigen. This demonstrated that the SIINFEKL peptide was actually undergoing cross-presentation.

Further studies of the HER2 protein to find specific peptides that can be directly combined with MHC I and elicit a large cytotoxic T cell response could lead to an effective vaccine targeting HER2 overexpressing tumor cells. The peptides naturally formed by HER2 break down within the proteosome and lysosome could be examined to determine which peptides are responsible for the pre-existing immunity found in patients with HER2 overexpressing breast cancer. Peptides not normally formed by the natural processing of HER2 could also be developed to elicit a new immune response to less dominant HER2 antigens. These less dominant antigens would be less likely to cause self-reactivity as suggested by Behrnard *et al.* (2002). There may also be a larger repertoire of T cells specific to these less dominant antigens, since they are less likely to be eliminated by negative selection to self antigens because the process is controlled by the more dominant antigens (Behrnard *et al.*, 2002). The self-reactivity observed as

cardiac dysfunction in treatment with trastuzumab may be avoided through the design of such a vaccine.

Glycosylation of the SIINFEKL peptide also appeared to slightly increase presentation when compared to the non-glycosylated SIINFEKL at the highest concentration. The β -gal production approached significance, with a p value of 0.078. These observations demonstrated the combination of the increased cross-presentation observed from the specific peptide along with glycosylation has the possibility to be developed into a very effective vaccine agent to a target cytotoxic T cell response to a tumor antigen.

The SIINFEKL peptide showed significant β -gal production using all methods of presentation assayed (the RPMI method, super-activating with ionomycin and super-activating with interferon- γ), the super-activation with ionomycin method however was the most effective. SIINFEKL presented by super-activation with ionomycin gave significantly greater β -gal production compared to the other two methods. This also demonstrated that the SIINFEKL peptide stayed complexed with the MHC I for an extended period of time, since the B3Z cells were able to be activated to produce β -gal 48 hrs after the initial presentation of SIINFEKL. This method gives an important insight into the formation of an ex-vivo vaccine against HER2. It suggests that supplementing the vaccine with ionomycin when pulsing the antigen presenting cells with the desired antigen may improve cross-presentation.

In another attempt to increase cross-presentation, the antigens were also presented in the presence of GM-CSF. The EVI antigen conjugated to GM-CSF, EVIrGM,

showed significant β -gal production compared to control at its highest concentration. This was one of the first experiments performed for this project, and was most likely before the B3Z cells become completely unresponsive to SIINFEKL presentation in the MHC I due to a decrease in the number of TCRs. It was hypothesized that the GM-CSF was able to bind the GM-CSF receptor on the cell surface, and the EVI was internalized along with the GM-CSF and its receptor, thereby cross-presenting the EVI peptides.

While the EVIrGM was able to be cross presented, EVI supplemented with GM-CSF, but not conjugated to it, did not give significant β -gal production. One study by Knutson *et al.* (2002) attempted to use GM-CSF as an adjuvant to a HER2 vaccine. While an initial immune response to the vaccine was detectable in two out of four patients, the magnitude of the response was low and short lived. The immunity was not detectable five months after the final vaccination. The observations of this study along with the results of this project, support the further study of GM-CSF conjugation to an antigen or peptide, rather than using it as an adjuvant. Conjugation to the peptide has demonstrated to increase the amount of cross-presentation and elicit an improved cytotoxic T cells response.

In an attempt to deliver the proteins directly into the cell and therefore increase cross presentation, several transfection agents were assayed. The only one to demonstrate significant increase in the presentation of SIINFEKL in the MHC I, as measured by β -gal production in B3Z cells, was the Polyplus-transfection agent PULSin. In combination with OVA, it was able to cause significantly greater β -gal production when compared to the control. The PULSin agent also had no significant effect on cell

viability when compared to the control. This demonstrated the possibility of transfection agents as adjuvants to vaccine antigens to target the antigen for MHC I presentation and activation of a cytotoxic T cell response.

Since the B3Z cells are specifically detecting SIINFEKL presented in the MHC I, and not specific HER2 peptides, this may not be the best model system to represent a HER2 antigenic vaccine. Other HER2 antigens may be processed by the splenocytes and presented more efficiently than the SIINFEKL peptide but are not measured using the B3Z assay. Since it has been demonstrated that only 6% of the possible SIINFEKL peptides that can be formed from the proteolysis of OVA actually stay intact (Rock et al., 2004), it is likely this same ratio would occur from processing EVI. While it appears that most of the SIINFEKL peptide is destroyed during processing, there may be other peptides within the HER2 portions of EVI that form at higher frequencies and are being presented more efficiently by the splenocytes.

An advantage of this model is that it directly measures the ability of the presented antigen to activate cytotoxic T cells. It is important for the desired antigen to not only have efficient presentation, activating more antigen presenting cells, but the presented antigen must also be able to activate specific cytotoxic T cells. In that respect, the model is a good representation of the specific cytotoxic T cell populations that exist within the immune system. It is also difficult to measure the specific antigen presentation in the MHC I directly on the splenocytes, as demonstrated by the lack of detection using the ELISA method. A model looking at IL-2 production by splenocytes

presented with specific peptide antigens from HER2 may be a more effective representation of a HER2 specific response.

The system has also been shown to assay the number of T cells activated, rather than just increasing the amount of β -gal produced by single cells. Initial studies after the formation of the B3Z cells showed that increasing stimulus increased the fraction of cells expressing β -gal, but not the level of lacZ activity per cell (Kattunen & Shastri, 1991). This gives the model physiological relevance as it mimics the mounting of a cytotoxic T cell response by activating a population of specific T cells.

In conclusion, this project has provided insight into possible mechanisms for increasing cross-presentation of tumor antigens. Glycosylation was demonstrated to be an effective tool in increasing the cross presentation of the HER2 protein EVI. Other approaches such as conjugation to GM-CSF or delivery of the protein into the cell by a transfection agent demonstrate the possibility of improving antigen cross-presentation. This project has also demonstrated the importance of finding a specific peptide to target within an antigenic protein in order to more efficiently stimulate cross-presentation and activation of cytotoxic T cells. The observations of this project provide insights into the optimal antigenic conditions to consider when forming an immunogenic therapy for HER2 overexpressing breast cancer.

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APPENDIX A:
LETTER OF APPROVAL FOR THE USE OF VERTEBRATE ANIMALS IN RESEARCH FROM
NORTHERN MICHIGAN UNIVERSITY'S DEAN OF GRADUATE STUDIES



Continuing Education & Sponsored Programs
1401 Presque Isle Avenue
Marquette, MI 49855-5325

May 25, 2007

TO: Robert J. Winn
Biology Department

FROM: Cynthia A. Prosen, Ph.D. 
Dean of Graduate Studies & Research

RE: **Application to use Vertebrate Animals**
Application # IACUC 060
Approval Period: April 11, 2007-April 30, 2010

The Institutional Animal Care and Use Committee approved your application for your project to use vertebrate animals in research entitled "Antigen Presentation by Spleenocytes and Dendritic Cells".

If you have any questions, please contact me.

kjm

cc: Biology Department

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